GLYCO 21

XXI International Symposium on Glycoconjugates

Abstracts

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Professor Jeffrey ESKO

Jeffrey D. ESKO, Ph.D., M.D. (h.c) is a Professor of Cellular and Molecular Medicine (cmm.ucsd.edu) and Co-Director of the Glycobiology Research and Training Center (grtc.ucsd.edu) at the University of California, San Diego. Dr. Esko received his Ph.D. in Biochemistry at the University of Wisconsin in Madison. After an independent fellowship at the Molecular Biology Institute at the University of California, Los Angeles, he moved to the University of Alabama at Birmingham and then to Department of Cellular and Molecular Medicine at the University of California, San Diego in 1996 to help build a program in Glycobiology. Work in his laboratory focuses on the structure, biosynthesis, and function of proteoglycans, including structural studies of heparan sulphate by mass spectrometry, application of genome-wide methods to identify genes involved in heparan sulphate assembly and lysosomal turnover, analysis of guanidinylated glycosides that act as molecular transporters, studies of proteoglycans in lipoprotein metabolism, and analysis of proteoglycans in modulating vascular permeability (eskolab.ucsd.edu). His work is supported by grants from the National Institutes of Health and the private sector. Dr. Esko has served on the numerous editorial boards and scientific boards and is currently on the board for Journal of Cell Biology and as an Associate Editor for Glycobiology. He was past President of the Society for Glycobiology, past Director of the Biomedical Sciences Graduate Program at UCSD (biomedsci.ucsd.edu), and he cofounded Zacharon Pharmaceuticals, Inc (www.zacharon.com). His work has been recognized by the Karl Meyer Award, the highest honor from the Society for Glycobiology, a MERIT Award from the National Institutes of Health, and an honorary degree from the University of Uppsala. In 2011, he is the recipient of the International Glycoconjugate Organisation Award.

Gerald W. Hart
The Johns Hopkins University,
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Plenary Lectures

001: Heparan sulfate proteoglycans as receptors and coreceptors (IGO Award Lecture)

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Heparan sulfate proteoglycans (HSPGs) are glycoproteins, with the defining characteristic of containing one or more covalently attached heparan sulfate (HS) chains, a type of glycosaminoglycan (GAG). Cells elaborate a relatively small set of HSPGs (~17), which show strong conservation through evolution. Much of the early work in the field concentrated on composition (size, chain number, and structure of the HS chains), biosynthesis, and binding properties, which led to a large list of potentially relevant ligands and interactions. In 1985, the first somatic cell mutants altered in HSPG expression were identified, which allowed functional studies in the context of a cell culture model. A decade later, the first HSPG mutants in a model organism *(Drosophila melanogaster)* were identified, which was followed by identification of mutants in nematodes, tree frogs, zebrafish, and mice. A particularly interesting area of concentration concerns the biological function of rare modifications of heparan sulfate. Biochemical and genetic approaches are underway to identify novel ligands and genes involved in positioning of sulfate groups along the chain. Other studies focus on the action of cell surface HSPGs as coreceptors, in particular their capacity to dock with signaling receptors that modulate vascular biology. HSPGs also act as adsorptive endocytic receptors, resulting in uptake of bound ligands. For example, hepatic HSPGs mediate the clearance of triglyceride-rich lipoprotein remnants, working in parallel with members of the LDL receptor family. When HSPGs enter the cell, they undergo lysosomal catabolism. Defects in enzymes involved in heparan sulfate turnover result in mucopolysaccharidoses (MPS) characterized by lysosomal storage and profound developmental and physiological effects. Efforts are underway to improve diagnosis and therapy for MPS based on diagnostic carbohydrate biomarkers and techniques to exploit HSPGs turnover for delivery of lysosomal enzymes to cells.

002: Biological roles of vertebrate glycans: a look back over the decades

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The structure, analysis and biochemistry of carbohydrates were matters of much scientific prominence in the first half of the 20th century, garnering several Nobel prizes and attracting much attention to their roles in metabolism, cell wall structure, cell surface antigens and microbe-host interactions. The International Symposium on Glycoconjugates was a natural outcome of such exciting discoveries. However, the development of viable lectin-resistant animal cell lines with major defects in glycosylation raised questions about whether complex glycans had specific functions intrinsic to the vertebrate organism itself. The discovery of the Ashwell receptor in 1969 and specific effects of heparin on antithrombin provided indirect evidence that complex glycans might carry out such functions. While many complex glycan biosynthetic pathways were also being elegantly worked out, there still remained no direct proof that glycans played intrinsic biological roles within vertebrate systems. As a trainee, I was fortunate to play a small part in the discovery by Kornfeld, Von Figura and others about 3 decades ago, that mannose 6-phosphate residues on lysosomal enzymes appeared critical for targeting of newly synthesized enzymes to the lysosome. This proposed biological role was confirmed by the elucidation of human genetic diseases in which the pathway was affected. The following decades have provided a flood of information regarding other biological roles of glycans in vertebrate systems, and the suggestion that "all of the theories are correct, but exceptions to each can be found" has proven correct. I will provide an outline of this historical overview and then focus on examples of specific biological roles of sialic acids intrinsic to vertebrates, including their contribution to selectin ligands, to neuronal stability and plasticity, and to the recognition of self by the Siglec family of sialic acid-recognizing Ig-like lectins. Finally, I will discuss multiple changes to sialic acid biology during human evolution and the potential implications for human diseases.

003: Regulation of spermatogenesis by complex N-glycans (Company of Biologists Lecture)

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In database searches to identify novel glycosyltransferase genes we discovered NM_026233.2 that encodes a physiological inhibitor of N-acetylglucosaminyl-transferase I (GlcNAcT-I or Mgat1). We termed this activity GlcNAcT-I Inhibitory Protein (GnT1IP). When expressed in cultured cells, membrane-anchored GnT1IP inhibits GlcNAcT-I activity but not other glycosyl-transferases such as GlcNAcT-III or galactosyltransferases. Inhibition of GlcNAcT-I causes cells to express mainly oligomannosyl N-glycans and to become resistant to the lectin L-PHA. In vivo, GnT1IP is expressed almost exclusively in testicular germ cells and expression of the

membrane-anchored form, GnT1IP-L, is tightly regulated during spermatogenesis. To determine if the altered N-glycan complement induced by GnT1IP might affect germ/Sertoli cell interactions, binding assays were performed using Sertoli cell lines TM4 and 15P-1. Cells overexpressing GnT1IP-L were found to bind more tightly to fixed Sertoli cells in a monolayer compared to control cells expressing complex N-glycans, indicating a potential role for oligomannosyl N-glycans in the binding of germ cells to Sertoli cells. In complementary experiments we are investigating roles for complex N-glycans by deleting Mgat1 in the testis using a variety of transgenes expressing Cre recombinase in germ or Sertoli cells. Deletion of Mgat1 in spermatogonia was found to cause a block in spermatogenesis that is currently being characterized. Therefore, while the regulated expression of oligomannosyl N-glycans may facilitate germ/Sertoli cell interactions that would persist in the absence of Mgat1, the subsequent generation of complex N-glycans appears to be essential for spermatogenesis. This work was supported by NIH grants RO1CA030645 and RO1CA036434 to PS.

004: Biological function of polylactosamine chains in the immune system

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Polylactosamine (Poly-*N*-acetyllactosamine) structure is a fundamental structure of carbohydrate chains and carries a lot of biofunctional carbohydrate epitopes. To investigate biological function of polylactosamine, we analyzed two knockout mouse strains of polylactosamine synthase-deficient mice.

β1,3-*N*-acetylglucosaminyltransferase (β3GnT) 2 is a major polylactosamine synthase. Firstly, we generated and examined *B3gnt2*-deficient (*B3gnt2*$^{-/-}$) mice. The amount of polylactosamine on glyccoproteins was markedly reduced in *B3gnt2*$^{-/-}$ mice. *B3gnt2*$^{-/-}$ lymphocytes showed hyperactivation via stimulation on cell surface glycoproteins, such as TCR/CD28 or BCR molecules. These results indicate that polylactosamine on N-glycans may play important roles in controlling the assembly and stabilization of the receptor complexes. Moreover, β3GnT5 (*B3gnt5*) is a lactotriaosylceramide synthase that synthesizes a precursor structure for lacto/neolacto-series glycosphingolipids (GSLs) containing polylactosamine structure. We generated and examined *B3gnt5*-deficient (*B3gnt5*-/-) mice. On various analyses, we found that BCR-related proteins, such as the BCR, CD19, and etc, were enriched into GEM. *B3gnt5*-/- B cells showed hyperactivation via BCR stimulation. Our results suggest that polylactosamine on GSLs may play important roles in controlling the clustering and stabilization of GEMs as a platform of signal transduction, and tether specific proteins, such as the BCR-related molecules, to the GEMs. We think that polylactosamine chains on glycoconjugates are the important factors determining thresholds for in vitro immune responses, at cellular level.

However, *B3gnt2*$^{-/-}$ mice showed lower responses in contact hypersensitivity (CHS) in vivo, at whole-body level. We found that neutrophil infiltration into inflamed tissue was decreased in *B3gnt2*$^{-/-}$ mice. We observed reduction of selectin ligands expression on *B3gnt2*$^{-/-}$ neutrophils. These selectin ligands, which were carried on polylactosamine chains, were down-regulated on some glycoproteins of *B3gnt2*$^{-/-}$ neutrophils. These findings may explain the lower responsiveness of contact hypersensitivity. Polylactosamine also influences the expression of terminal carohydrate epitopes and other immune reaction, such as leukocyte trafficking, resulting in reduction of CHS response on individual level. These finding indicates that polylactoamine has important roles in immunological biofunctions. This work was supported by New Energy and Industrial Technology Development Organization (NEDO) in Japan.

005: Lysosomal disorders: insights into fundamental cell biology

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Lysosomal storage diseases are a group of over 50 human disorders that result from defects in any aspect of lysosomal function. The majority present in infancy/childhood and commonly have a neurodegenerative clinical course. They are typically inherited as autosomal recessive traits. Most lysosomal disorders are the result of mutations in genes that encode acid hydrolases, resulting in the storage of the specific enzyme's substrate(s) in the acidic compartment. However, we now know that a sub-group of these diseases are the result of defects in lysosomal membrane proteins, the majority of which are currently of unknown function. Studying these disorders is shedding considerable light on membrane protein functions in the lysosomal system and how these proteins affect cellular homeostasis more generally. Understanding disease pathogenesis in these diseases is therefore providing novel insights into aspects of fundamental cell biology that would have been hard to discover in the absence of these disorders. Examples of recent developments in this area will be presented, along with the therapeutic implications of these findings for treating these devastating diseases.

006: Modulation of the immune system by parasitic helminths: the role that glycans might play (ESF Lecture)

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Chronic helminth infections have strong effects on the immune system: they skew responses towards Th2 and regulatory cells. To understand the molecular mechanisms whereby the immune system is modulated, it would be important to identify and study parasite-derived molecules with immune modulatory activities. Parasitic helminths express special array of glycans that are thought to play an important role in their interaction with the mammalian host; some with the ability to modulate the immune system. Interaction of C typle lectins (CTLs) such as DC-SIGN, the Mannose Receptor and Dectin-1 or 2 with their ligands is known to have potent immunological effects. A number of molecules from helminths have now been identified that use specific CTLs to skew immune responses. Strong modulation of the immune system by helminths can have important consequences for responses to unrelated antigens and for clinical outcomes and therefore well defined parasite derived molecules may have therapeutic potential for a number of diseases.

007: The glycobiology of muscular dystrophy

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A subset of muscular dystrophies (the dystroglycanopathies), which are often associated with CNS and ocular defects, are caused by mutations in genes necessary for glycosylation of the alpha-subunit of dystroglycan. Dystroglycan binds extracellular matrix proteins such as laminin and agrin. Correct glycosylation of dystroglycan is necessary for this ligand-binding activity. Alpha-dystroglycan contains a central mucin-like domain that is highly decorated with O-glycans, including unusual O-mannosyl structures. Recent data implicate a requirement for an O-linked phosphomannose glycan. Recessive mutations in at least six genes (POMT1, POMT2, POMGnT1, Fukutin, FKRP and LARGE), several of which are known to play a role in synthesis of O-mannose glycans, are causative for dystroglycanopathy. My group has focused on the study of the LARGE gene, which is predicted to encode a protein with two glycosyltransferase catalytic domains. The function of LARGE is evolutionarily very ancient; orthologues of this gene are found in almost all animal genomes, including those of cnidarians and sponges. LARGE activity is necessary for functional ligand-binding by dystroglycan. Over-expression of the enzyme is able to induce hyperglycosylation of dystroglycan and concomitant laminin-binding, even in cells with mutations in other dystroglycanopathy genes. Thus, LARGE represents an important potential therapeutic target. Dystroglycan also acts as a pathogen receptor and arenavirus-binding the protein requires the presence of the same glycan structures as laminin-binding. Thus, determination of the glycan structurs of dystroglycan is of medical importance. Remarkably, this glycosylation pathway appears to be highly specific, as dystroglycan is the only substrate identified thus far.

008: Remodeling of GPI anchors in the ER before and after attachment to proteins: mechanisms and functions

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Glycosylphosphatidylinositol (GPI) is synthesized from PI in the ER via at least eleven reaction steps and attached en bloc to proteins. Biosynthesis of GPI is not a simple assembly of building blocks on PI but involves structural remodeling in both lipid and glycan parts before and after attachment to proteins. In this talk, I deal with mechanisms and functions of remodeling reactions occurring in the ER. (1) The major form of GPI anchors in mammalian cells contains 1-alkyl-2-acyl glycerol in their PI moiety. Biosynthesis of GPI is initiated with diacyl PI and remodeling to 1-alkyl-2-acyl form occurs in the third intermediate, glucosaminyl-(inositol-acylated)PI. CHO cells defective in the peroxisomal alkylphospholipid biosynthetic pathway generated only diacyl but not 1-alkyl-2-acyl GPI-anchors. This remodeling may require a putative donor 1-alkyl-2-acyl phospholipid. In peroxisomal disorders, Zellweger syndrome and rhizomeric chondrodysplasia punctata (RCDP), incorporation of enzymes for alkylphospholipid biosynthesis into the peroxisome is defective. We showed that generation of 1-alkyl-2-acyl GPI-anchors is defective in CHO cells defective in PEX genes responsible for Zellweger syndrome and RCDP and in cells from patients with these diseases. A lack of 1-alkyl-2-acyl GPI-anchors might account for some of the clinical symptoms. (2) After attachment of GPI to proteins, two remodeling reactions occur, removals of inositol-linked acyl chain by PGAP1 and of ethanolaminephosphate side-branch from the second mannose by PGAP5. These remodelings are required for efficient sorting of GPI-anchored proteins into the ER-exit sites and association with p24 proteins. p24 family of proteins acts as a cargo receptor for GPI-anchored proteins in packaging into COPII-coated transport vesicles.

009: Surface glycosylation in *C. elegans*: impacts on bacterial infection, movement, mating, development and drug sensitivity

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The nematode *Caenorhabditis elegans* provides a powerful model system for investigating biological functions of carbohydrate modification. We have found that infection of *C. elegans* by a variety of bacterial pathogens is dependent on normal surface glycosylation, mediated by transporters and enzymes expressed in ectodermal seam cells and/or intestinal cells. Selection for resistance to these pathogens yields mutations in multiple genes encoding glycosylation enzymes, most of which are compatible with viability but result in detectable alterations to O-glycans and altered lectin staining. Complete knockout of one gene, *bus-8*, is embryonic lethal owing to failures of epithelial cell migration. Null mutants of most of the other genes are viable and resistant to one set of bacterial pathogens, but become lethally hypersensitive to other surface pathogens, thereby revealing selective trade-offs in surface glycosylation. Many of these viable mutants also exhibit abnormalities in other interfacial properties, such as locomotory traction and conspecific recognition during mating behavior. Some mutants also display greatly increased sensitivity to many low-molecular weight drugs, indicating that the nematode glycocalyx provides a major permeability barrier and protection against toxic compounds. These hypersensitive mutants increase the utility of *C. elegans* for high-throughput drug screening. They are also being used to investigate the complex carbohydrate surface coat, which constitutes the main interface between nematodes and their environment, both in free-living species such as *C. elegans*, and in parasitic nematodes of agricultural or medical importance.

010: Analytical glycobiology at high sensitivity: a decade of progress (Jean Montreuil Lecture)

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Since the pioneering studies of the 1980s, the field of glycobiology has expanded enormously, implicating various glycoconjugates in the ever-increasing number of important biological functions occurring in virtually all living organisms, both eukaryotic and prokaryotic. It has been increasingly recognized that glycans of different chemical structure are most typically the biological determinants in selective interactions through glycoproteins, glycolipids and proteoglycans. A direct structural and quantitative measurement approach to numerous glycome components is subsequently needed to understand the functional aspects of a given organism, or to complement the knowledge achieved through genomic studies and transcriptional profiling. Much overall progress in glycobiology realized during the last decade is due to the revolutionary changes in biomolecular mass spectrometry (MS) and its combinations with capillary separation techniques. Additionally, the newly developed glycan array technologies provide effective ways to study carbohydrate-binding proteins. Sensitive structural glycomic measurements may involve different modes of MS, capillary electrophoresis, or capillary liquid chromatography, once either N-linked or O-linked oligosaccharides are quantitatively released (enzymatically or chemically) from their respective glycoproteins. For effective profiling by MALDI-MS, it is advisable to convert the measured glycans to their methyl derivatives, as the permethylation step covers the neutral and acidic oligosaccharides and improves MS data. When working with very small biological samples, well over 100 N-glycan structures can be profiled in quantitative fashion to account for glycosylation differences in health and disease or other comparative glycomic investigations. Consequently, structural identification of glycan-based disease biomarkers has become possible, as documented by the recent examples from cancer research. The serum glycoprotein analyses of low-microliter volumes have been developed for the benefits of early diagnosis and prognostic measurements. Delving deeper into the glycomic differences between the "normal" and pathological states involves preconcentration and measurements of highly sialylated and fucosylated glycans. Ultimately, quantitative glycomic measurements must be related to unique proteins through the emerging tools of high-sensitivity glycoproteomics.

011: Lipid A biosynthesis and modification in Gram-negative bacteria

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The lipid A moiety of lipopolysaccharide forms the outer monolayer of the outer membrane of most gram-negative bacteria. *Escherichia coli* lipid A is synthesized on the cytoplasmic surface of the inner membrane by a conserved pathway of nine constitutive enzymes, several of which are excellent targets for the design of new antibiotics and are amenable to high-resolution structural studies. Following attachment of the core oligosaccharide, nascent core-lipid A is flipped to the outer surface of the inner membrane by the ABC transporter MsbA, where the O-antigen polymer is attached. Assembly into the outer membrane requires a second ABC transporter and two additional outer membrane proteins. Diverse covalent modifications of the Kdo-lipid A domain of lipopolysaccharide may occur during its transit from the inner

membrane to the outer membrane. Lipid A modification enzymes are therefore excellent reporters for lipopolysaccharide trafficking within the bacterial envelope. Although lipid A modification enzymes are not required for growth and are not conserved in all Gram-negatives, they can usually be reconstituted by heterologous expression in *E. coli* or *Salmonella*, enabling the construction of novel strains with altered Kdo-lipid A structures and attenuated virulence.

012: Carbohydrate chemistry and drug discovery

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Protein glycosylation is the most complex post-translational process; more than 90 percent of human proteins are glycosylated. The significance of glycosylation at the molecular level is however not well understood, and as such the pace for the development of carbohydrate-based drug discovery and diagnosis is relatively slow. It is thus important to develop new tools to study the effect of glycosylation on the structure and function of proteins and other biologically active molecules. This lecture will focus on the development of new methods for the synthesis of homogeneous glycoproteins with well defined glycan structure, glycoarrays for the high-throughput analysis of protein-glycan interaction and design of click-induced fluorescent probes for use to identify new cancer biomarkers for diagnosis and drug discovery. New glycoprotein vaccines have been designed and developed to tackle the problems of flu and breast cancer.

013: Enzymes of the Golgi apparatus involved in plant cell wall synthesis (ESF Lecture)

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Plant cell wall polysaccharides have important roles in growth, development, and resistance to pathogens. They are also of enormous importance in industry and agriculture, being substantial components of human and animal food, and determine the yield and properties of timber, paper and pulp. Recently, there has been great interest in using the polysaccharides as a source of renewable bioenergy, for example by degrading them to monosaccharides and fermenting to ethanol. In addition to cellulose, which may constitute 40% of the cell wall, hemicellulosic sugars from xylan and mannan are also an important bioenergy target, and may constitute a further 30% of the mass. To utilise them effectively, we need to understand how they are made, so that their structure and quantity can be optimised. With this knowledge, strategies can be devised to improve the release of sugars from the cell wall for fermentation. To discover proteins involved in the synthesis of these glycans, we have been analyzing the protein composition of the Golgi apparatus by developing quantitivative proteomics tools. We have identified putative glycosyltransferases, sugar transporters and other novel proteins in the Golgi apparatus in *Arabidopsis*. We prioritise the study of candidates by integrating transcriptomic and proteomic datasets to predict function. We are studying the corresponding mutant plants using an enzymatic polysaccharide profiling technique PACE, with mass spectrometry, which together reveal structure and quantity of oligosaccharides released by cell wall digestion. Plants with altered cell wall polysaccharides have recently been identified, and show unexpected phenotypes which may be useful for biorefining and bioenergy.

014: Principles of N-linked protein glycosylation

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N-linked protein glycosylation is the most frequent post-translation modification in eukaryotic cells. In the Endoplasmic Reticulum, an oligosaccharide is assembled on the lipid carrier, dolichylpyrophosphate and transferred to selected asparagines residues of translocated polypeptide chains. In most of the eukaryotes, the oligosaccharide is $Glc_3Man_9GlcNAc_2$, however, in protozoa, simpler structures are also found. The covalent linkage of the oligosaccharide is performed by the oliogosaccharyltransferase (OST), a complex enzyme that is composed of eight different subunits, all of them membrane embedded proteins. N-glycosylation of proteins also exists in prokaryotes and detailed studies of a bacterial system discovered in *Campylobacter jejuni* showed that the bacterial and the eukaryotic process are homologous pathways that are based on the same principles: both pathways utilise isoprenoid lipids as carriers for the assembly of the oligosaccharide in the cytoplasm (requiring a translocation of the oligosaccharide across the membrane) and the AsN-X-Ser/Thr is the protein acceptor sequence in both cases. The functional analysis of oligosaccharyltransferases from bacteria, protozoa and yeast revealed the basic concepts of the N-glycosylation process. A mechanistic model for N-glycosylation, based on the detailed structural information of the bacterial oligosaccharyltransferase, now identifies the molecular parameters that define these unique properties of the N-glycosylation process.

Development & Differentiation I (Monday)

015: Neural functions of sialylation in *Drosophila* (Keynote)

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Sialic acids represent a family of acidic sugars that predominantly occupy termini of carbohydrate chains and affect a plethora of biological functions in vertebrate organisms. While twenty different sialyltransferases function in mammals, Drosophila has a sole vertebrate-type sialyltransferase, DSiaT, with significant homology to its mammalian counterparts. The expression of DSiaT is restricted to a subset of CNS neurons throughout all developmental stages. To shed light on the role of sialylation in Drosophila, we inactivated DSiaT in vivo by gene targeting and studied the phenotypes of DSiaT mutants using a combination of behavioral, immunolabeling, and electrophysiological approaches. We found that DSiaT mutations significantly affect the functions of the nervous system, resulting in locomotor abnormalities and temperature-sensitive paralysis. Our experiments demonstrated that DSiaT mutants have defects of neuromuscular junction development and physiology. Our study also provided the evidence that sialylation regulates neuronal excitability and modulates the function of a voltage-gated sodium channel. These data revealed a novel, nervous system-specific function of sialylated N-glycans, and suggested that this function is evolutionarily conserved between Drosophila and mammals, thus shedding light on one of the most ancient functions of sialic acids in metazoan organisms.

016: Disorganization of muscle ultrastructure along with defective motor function and increased apoptosis of myoblasts in *Drosophila* model for the Walker-Warburg syndrome

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Walker-Warburg syndrome is a progressive muscular dystrophy with various kinds of symptoms such as muscle weakness and occasional seizures. The genes of protein O-mannosyltransferases 1 and 2 (*POMT1* and *POMT2*), fukutin, and fukutin-related protein are responsible for this syndrome. In our previous study, we cloned Drosophila orthologs of human *POMT1* and *POMT2* and identified their activity (JBC, 279, 42638-42647(2004)). However, the mechanism of onset of this syndrome is not well understood. Furthermore, little is known about the behavioral properties of the *Drosophila POMT1* and *POMT2* mutants, which are called rotated abdomen (*rt*) and twisted (*tw*), respectively. Therefore, we carried out various kinds of behavioral tests, described in detail the muscle structures, and analyzed the development of the muscles by using these mutants (PLoS One, 5, e11557 (2010)). The mutant flies exhibited abnormalities in heavy exercises such as climbing or flight but not in light movements such as locomotion. Defective motor function in mutants appeared immediately after eclosion and was exaggerated with aging. Along with motor function, muscle ultrastructure in the *tw* mutant was altered, as seen in human patients. Flies expressing RNAi for the *rt* gene had reduced lifespans. These findings clearly demonstrate that *Drosophila POMT* mutants are models for human muscular dystrophy. We then observed a high density of myoblasts with an enhanced degree of apoptosis in the tw mutant, which completely lost enzymatic activity. Therefore, we propose a novel mechanism for the development of muscular dystrophy: *POMT* mutation causes high myoblast density and position derangement, which result in apoptosis, muscle disorganization, and muscle cell defects.

017: Embryonic Lethality of β-1,4-Galactosyltransferase V-Deficient Mice

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We cloned human and murine β-1,4-galactosyltransferase (β-1,4-GalT) V-genes (1, 2), and have recently showed that murine enzyme is involved in the biosynthesis of lactosyceramide (Lac-Cer) but not of N-glycans by in vivo studies (3). Namely, an enzymatic activity toward glucosylceramide, and amounts of Lac-Cer and its derivative GM3 were remarkably reduced in mouse embryonic fibroblast (MEF) cells isolated from β-1,4-GalT V-deficient mouse embryos when compared to those from control animals. Contrary to our previous studies (4), no significant changes in β-1,4-GalT activities toward GlcNAcβ-S-pNP and in amounts of individual N-glycans were obtained between control and mutant MEF cells.

As we described previously, the mutant mice die at the mid-embryonic stages with severe growth retardation, particularly of brain (5). Immunohistochemical studies showed that brains of the mutant mouse embryos at E10.5 react to an antibody against protogenin expressed specifically in neural progenitor cells but not to an antibody against βIII-tubulin expressed in differentiated neural cells, while brains from control mouse embryos at E10.5 react to an antibody against βIII-tubulin but not to an antibody against protogenin. In the case of an

antibody against Nestin expressed in neural stem cells, brains of the mutant mouse embryos contained much more Nestin-positive cells than those of control animals at E10.5. These results indicate that differentiation from neural progenitor cells to neural cells is impaired by the lack of Lac-Cer and its derivative glycolipids in β-1,4-GalT V-deficient mice. Further studies are necessary for elucidating what kinds of glycolipids are important for early development of mouse brain.

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018: Muscle defects in flies with disrupted Golgi function

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Golgi-linked glycosylation of secreted proteins is important for many cellular functions including signalling, cell attachment or protein sorting. However, the exact functional determinants of the generated oligosaccharides – also called glycans – are mostly unknown. To engineer a model system for specific glycan defects we have perturbed the conserved oligomeric Golgi (COG) complex that is known to modulate glycosylation in *Drosophila melanogaster*. Indeed, mutation of the COG5 subunit in four-way stop (*fws*) flies causes glycosylation defects as assessed by lectin blotting of fly tissues. The *fws* mutation also causes a range of muscle defects including diminished or absent flight, reduced jumping ability and reduced walking speed. Electron microscopic analyses of the indirect flight muscles show aberrant attachment sites between muscle and tendon cells. This indicates that the potential glycosylation defects caused by COG malfunction could be affecting the extracellular matrix during muscle development. Human COG mutations have been shown to cause congenital glycosylation disorders with pleiotropic developmental effects. A fly model could in the future help discriminate between different tissue-specific defects and could inform the human disease case.

Development & Differentiation II (Monday)

019: Glycans in nervous system development, regeneration and synaptic plasticity (Keynote)

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The functional roles of glycan chains attached to adhesion molecules have been recognized to be crucial for nervous system development in many if not most animal species. The first reported example of a functionally important glycan was α2,8 polysialic acid attached to 'embyronic' NCAM. It was thought, that due to its large hydration volume, it increased the intercellular space to facilitate migration of neural cells and extension of their processes. Receptors for this glycan were subsequently found to participate in these events. Interestingly, this glycan is one of the factors mediating regeneration of the injured peripheral nervous system. In the central nervous system, it beneficially overcomes the limited capacity for regeneration in the adverse tissue environment of the adult mammalian brain and spinal cord. In addition, this glycan is involved in altering the activity of synapses by interaction with the NR2B subtype of the glutamatergic NMDA neurotransmitter receptor, thus regulating learning and memory processes. Another unusual glycan epitope is HNK-1 (human natural killer cell) which also plays an important role in synaptic activity by interacting with the GABA B neurotransmitter receptor. It is an important determinant in regeneration of the adult peripheral nervous system of mice and monkeys by specifying regrowth of severed axons of spinal motorneurons allowing them to choose the correct nerve branch when given a choice between motor and sensory nerves. We have used glycomimetic peptides to investigate functions of these glycans, as peptides can be designed to be more stable and more readily available than their natural counterparts. Lewis x and oligomannosidic glycans, as well as glycomimetic peptides, have also yielded insights into their functions in the nervous system. Efforts are underway to abstract these glycans into small organic molecules by screening libraries of organic synthetic and chinese traditional medicine compounds. It is expected that our studies will contribute to approaches towards clinically oriented translational research.

020: Essential roles of gangliosides in the protection of inflammation and neurodegeneration via the regulation of lipid rafts: elucidation by a series of ganglioside-deficient mice

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Gangliosides are now considered to be important in the maintenance and repair of nervous tissues. Recently, novel roles of gangliosides in the regulation of complement systems were demonstrated, i.e. complement activation was caused in GM3-only mice, resulting in the inflammation and subsequent neurodegeneration. Dysfunctions of complement-regulatory proteins such as CD55 and CD59

seemed to be main factors to trigger complement activation. Here we compared complement activation, inflammatory reaction and disruption of glycolipid-enriched microdomain (GEM)/rafts among various mutant mice of ganglioside synthases, i.e. GM2/GD2 synthase KO, GD3 synthase KO, double knockout (DKO) of these two enzymes as well as wild type (WT). Cerebellar weights of DKO were significantly lower than those of wild type. Immunoblotting of fractions from sucrose density gradient ultracentrifugation revealed that lipid raft markers such as caveolin-1 and flotillin-1 tended to disperse from the raft fractions with intensities of DKO > GM2/GD2 synthase KO > GD3 synthase KO > WT. Among GPI-anchored proteins, DAF and NCAM tended to disappear from the raft fraction. Up-regulation of complement-related genes, deposits of C1q, proliferation of astrocytes and infiltration of microglia also showed similar gradual severity depending on the defects in ganglioside compositions. In the expression of inflammatory cytokines such as IL-1β and TNFα, only DKO showed definite up-regulation. These results indicate that destruction of GEM/rafts is caused by ganglioside deficiency with gradual intensity depending on the degree of defects of their compositions. Analyses of triple KO in which complement systems were inactivated as well as two ganglioside synthases revealed that complement activation plays main roles in the inflammatory reaction and neurodegeneration. These results suggested essential roles of gangliosides in the protection of complement activation, and also of neurodegeneration. These results also suggested the possibility to control the complement systems and neurodegenerative diseases such as Alzheimer diseases by manipulating ganglioside expression.

021: NGF induced PC12 cell neuritogenesis is promoted by heparanase via p38 MAPK signalling pathway

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Heparanase is a heparan sulphate (HS) degrading endoglycosidase participating in the degradation and remoldelling of the extracellular matrix (ECM) and the basement membrane (BM). Apart of its implication in inflammation and tumor angiogenesis and metastasis, heparanase also plays a critical role in central nervous system. In this report, we demonstrate that heparanase enhances nerve growth factor (NGF) induced neuritgenesis in the rat adrenal pheochromocytoma (PC12). The progress can be blocked by suramin, an inhibitor of heparanase. Overexpression of human heparanase results in activation of p38 phosphorylation and elevation of NGF induced neuritogenesis in PC12 cells, while heparanase knockdown impairs this effect. Interestingly, it seems that the neuritogenesis and the phosphorylation of p38 induced by NGF is not the enzyme activity issue since the mutated heparanase by replacing the active site Tyr^{156} with Ala, reveal the same extent as previously observed. Inhibition of p38 MAPK by its inhibitor, SB203580, neither of heparanase nor mutant heparanase shows the facilitative activity of neuritogenesis induced by NGF, meanwhile the heparanase knockdown cells can be restored by transfect with heparanase or mutant heparanase. Taken together, our results suggest that heparanase may promote the NGF induced neuritogenesis via the MAPK p38 signalling pathway independent of its enzyme acitvity.

022: Polysialic acid chains display attractive fields toward neuroactive molecules

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Polysialic acid (polySia) is a unique polymer that modifies the neural cell adhesion molecule (NCAM) spacio-temporally in brain. Recently, we demonstrated that polySia functions not only as an anti-adhesive molecule, but also as a reservoir molecule for neurotrophic factors such as BDNF (1). The latter is a new function of polySia that has not been described so far. To gain further insight into the new feature of polySia function, we examined if polySia had the ability to bind neurotransmitters, using frontal affinity chromatography. Among several neurotransmitters tested, polySia was shown to bind to the catecholamines such as dopamine (DA), norepinephrine, and epinephrine (2). In this study, we focused on FGF2 and asked if polySia directly interacted with FGF2 by the surface plasmon resonance and the native-PAGE methods. PolySia was found to make a complex with FGF2 differently in the size and the binding property to FGF receptors than heparan sulfate (HS), a well-known FGF2-binding glycosaminoglycan. Finally, we asked if BDNF, DA, and FGF2 were retained by polySia-NCAM biosynthesized by mutated polysialyltransferases (STXs) reported in schizophrenia patients. The mutated STXs showed impaired enzyme activities and synthesized a smaller amount of polySia with shorter chain length (DP) on NCAM. In the mutated STX-derived polySia-NCAM, binding with all these neuroactive molecules was significantly impaired. All these results indicate that regulation of the amount and DP of polySia on NCAM might be important for normal brain functions.

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Development & Differentiation III (Monday)

023: Glycosaminoglycans as regulators of stem cell differentiation (Keynote)

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Glycosaminoglycans (GAGs) are essential cofactors for many signalling molecules which regulate stem cell expansion and differentiation. Whereas many groups are investigating the protein components of these signalling complexes, the carbohydrate fraction is less well understood and therefore remains an under-appreciated factor in the design of stem cell expansion strategies or methods to direct differentiation. For the future differentiation of stem cells to the target cell type, systems must be scalable, fully-defined and xeno-free. We have improved the understanding of specific GAG epitopes in mouse ES cells, demonstrating that selected GAG saccharides can be used to influence specific signalling pathways during neural and mesodermal differentiation. Importantly, the sulphation pattern and size of saccharides required for neural differentiation were distinct from those required to drive the formation of alternative cell fates such as haematopoiesis. This suggests that GAG saccharides could be used in addition to protein additives in differentiation protocols designed to optimise the generation of therapeutically relevant cell types from stem cells. Chemoenzymatic production of xeno-free structurally-defined GAG oligosaccharides now allows the use of these compounds for control of cell signalling. Importantly, these compounds can also be generated on a scale and at a level of purity acceptable for inclusion in GMP stem cell differentiation protocols. We are combining this work with ongoing studies into the design of artificial cell environments where we have optimized three-dimensional scaffolds, generated by electrospinning or by the formation of hydrogels, for the culture of ES cells. By permeating these scaffolds with defined GAG oligosaccharides, we can control the mechanical environment of the cells (via the scaffold architecture) as well as their biological signalling environment (using the oligosaccharides).

024: Protein O-glycosylation in ECM formation and organogenesis

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Mucin-type O-glycosylation of proteins is an abundant post-translational modification that plays essential roles during eukaryotic development. Recent studies in *Drosophila melanogaster* have demonstrated that O-glycosylation influences the composition of the basal extracellular matrix (ECM), thereby affecting integrin-mediated cell adhesion during development. Here we examine the role of mucin-type O-glycosylation in basement membrane (BM) formation during mammalian submandibular gland (SMG) development. Expression analysis of staged SMGs, from embryonic day 12 (E12) to adulthood, demonstrated unique temporal expression patterns for 16 ppGalNAcT family members that are responsible for initiating O-linked glycosylation. Mice deficient for an isoform that was identified as being expressed most abundantly during early SMG development (ppGalNAcT-1) showed reduction in O-glycans specifically along the BM of embryonic day 12 (E12) SMGs, along with reductions in the major BM components (collagen IV, laminin α1 and perlecan). Interestingly, ppGalNAcT-1 deficient SMGs also displayed smaller initial end buds and reduced growth when compared to wild type. EdU labeling revealed reduced cell proliferation in ppGalNAcT-1 deficient E12 SMGs. Analysis of growth factors involved in SMG growth and development revealed reduced expression of Fgf1. As BM components are known to regulate expression Fgf1 and other growth factors responsible for SMG growth, we hypothesize that alteration in BM composition as a result of the loss of ppGalNAcT-1 is responsible for the reduced Fgf1 expression and cell proliferation observed. Our work demonstrates that O-glycosylation influences the composition of the extracellular matrix in mammalian organ development, with resultant effects on cell proliferation and organ growth. These results highlight a conserved role for O-glycosylation in the establishment of cellular microenvironments that have implications for the role of this protein modification in both development and disease.

025: Xylosyltransferases Involved in Modification of Notch EGF Repeats

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The extracellular domain of Notch consists of EGF repeats which are heavily modified with different O-linked glycans. The biological impact of O-fucosylation followed by elongation with N-acetylglucosamine has been well established, but there is little known on how O-glucose and its subsequent extension with two xylose residues influence the Notch signaling pathway. Until recently, not all genes involved in the generation of the Notch specific epitope xylose-xylose-glucose were known. In previous

studies we have identified two human genes GXYLT1 and GXYLT2 (glucoside-xylosyltransferase 1/2) encoding enzymes which are able to transfer the first xylose residue on glucosylated EGF repeats (Sethi et al.; 2010; J. Biol. Chem. 285). Now we have discovered a related human gene encoding a xyloside-xyloslytransferase (XXYLT) responsible for the transfer of the second xylose residue of the trisaccharide. The enzyme showed high donor-substrate specificity for UDP-xylose and requires a xylose-glucose disaccharide as acceptor in vitro. This is consistent with the observed enzymatic activity in vivo, where XXYLT only extends O-linked glucose-xylose residues on Notch EGF repeats and is not elongating the glycan further than the trisaccharide. Together with Rumi, the glucosyltransferase identified in Drosophila (Acar et al.; 2008; Cell 132), all enzymes involved in the O-glucosylation pathway are now known. This enables functional studies to decipher how these unusual carbohydrate modifications influence Notch signaling.

026: Leukemia inhibitory factor promotes trophoblast migration and invasion via uPA/uPAR-mediated FUT7 expression

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Leukemia inhibitory factor (LIF) is an essential cytokine which is involved in embryo development, implantation and extravillous trophoblast (EVT) migration and invasion; et al. We previously found that LIF up-regulated the expression of FUT7, while LIF antibody down-regulated FUT7 expression in mouse embryo. It is reported that urokinase plasminogen activator (uPA)/urokinase plasminogen activator receptor (uPAR) is detectable in the EVT cells. However, whether the regulation of FUT7 expression by LIF is mediated by uPAR is not known. In this study, we found that LIF activated uPA/uPAR system in human trophoblast cells (JAR), and facilitated trophoblast migration and invasion; while, PAI-1, an inhibitor of uPA, inhibited the expansion of trophoblast on human uterine epithelial cell RL95-2 monolayer. Overexpression of uPAR elevated the FUT7 expression and promoted trophoblast expansion. The results indicate that LIF promotes trophoblast migration and invasion via uPA/uPAR-mediated FUT7 expression. The work was supported by the grant NSFC: (31070729).

Carbohydrates and Disease I (Monday)

027: Mechanisms of basement membrane disruptions in congenital muscular dystrophies

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Brain malformations and retinal dysplasia in a subset of congenital muscular dystrophies (CMDs), dystroglycanopathies, are caused by disruptions of the basement membranes. Reduced extracellular matrix binding by the hypoglycosylated α-dystroglycan because of mutations in several glycosyltransferases is the underlying molecular defects. It is not known how the basement membrane becomes disrupted. We hypothesized that assembly of the basement membrane is reduced in rate thus causing a physically compromised basement membrane. To test this hypothesis, we analyzed assembly of extracellular matrix on cultured neural stem cells and physical properties of the mutant basement membrane by atomic force microscopy. Laminin assembly on the protein O-mannose β1,2-N-acetylglucosaminyltransferase 1 (POMGnT1) knockout neural spheres was reduced when compared to the wildtype. When incubated with Matrigel, extracellular matrix (ECM) molecules including all four major components of the basement membrane, laminin, collagen IV, perlecan, and nidogen co-aggregated. Rate of ECM aggregation was reduced on POMGnT1 knockout neural sphere as revealed by slower growth in aggregate size when compared to the wildtype. Immunofluorescence staining and proteomic comparison revealed that the mutant basement membrane exhibited compositional changes from the wildtype. Atomic force microscopic analysis revealed that the mutant basement membrane had reduced elastic modulus with surface topography showing bigger valleys than the controls. Thus, disruptions of the basement membrane in dystroglycanopathies may be caused by its weakened strength resulted from biochemical changes in composition and physical changes in structure and reduced assembly rate.

028: Fucosylation of plasma proteins is markedly decreased in maturity-onset diabetes of the young – proof of principle for the integrated genomic/glycomic approach to glyco-biomarker discovery

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Maturity-onset diabetes of the young (MODY) is a dominantly inherited form of non-insulin dependent diabetes caused by mutations in several genes. A subtype of MODY is caused by mutations in HNF1α, a nuclear transcription factor which appears to be one of the key regulators of metabolic genes. Recently we performed the first genome wide association analysis of the human plasma N-glycome and identified HNF1α as a master regulator of plasma protein fucosylation which promotes both de novo and salvage pathways for GDP-fucose

synthesis. Since even non-coding polymorphisms in the HNF1α gene have clearly detectable multiple effects on plasma protein fucosylation, we hypothesized that deleterious coding mutations in HNF1α should have even more profound effects and that antennary fucosylation of plasma proteins could be significantly decreased in HNF1α-MODY patients. Aiming to assess the potential value of decreased antennary fucosylation of plasma proteins as a biomarker in MODY we analyzed the plasma N-glycome in 61 patients with HNF1α-MODY, 77 patients with GCK-MODY, 70 patients with diabetes type 1 and 50 patients with diabetes type 2. Glycan profiles obtained by the analysis of over 3500 individuals from general population were used to determine reference values. The most prominent changes in HNF1α-MODY were the significant decrease in several HPLC peaks containing mainly antennary fucosylated glycans and the increase in peaks containing mainly glycans without antennary fucose. HNF1α-MODY patients could be nearly completely separated from all other studied groups on the basis of antennary fucosylation of plasma glycoproteins with Receiver-Operator Characteristic (ROC) curves approaching 90% specificity at 90% sensitivity. This finding represents an efficient proof of principle for the integrated glycomic/genomic model of biomarker discovery which uses genome wide association approach to identify genes relevant for protein glycosylation and then screen for specific changes in glycans in patients with a specific subtype of a complex disease associated with that gene.

029: Synthesis of Glycopeptides to Probe Glycosylation of α-Dystroglycan

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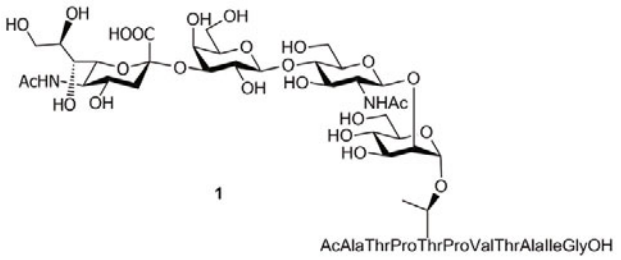
The extracellular glycoprotein α-dystroglycan (α-DG) is associated with congenital muscular dystrophy. α-DG is characterised by a unique type of glycosylation, namely O-mannosylation, a common post translational modification in yeast but rare in mammals. It is now well established that the biosynthesis of these O-glycans is affected in congenital forms of muscular dystrophies, but the exact biochemical mechanisms are still poorly understood. Here we describe the synthesis of diverse glycopeptides that have been isolated from α-DG and evaluate them as substrates for potential α-DG glycosyltransferases. The total synthesis of the unusual tetrasaccharide of α-DG (1) is described for the first time.



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030: Glycosylation in the Pathophysiology of Heritable and Acquired Disease (Keynote)

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Of the 34 monosaccharides thus far identified that are used in the enzymatic process of glycosylation among all cells and organisms, mammalian glycans are produced from only 9 of these monosaccharides. With this limitation of monosaccharides and the glycosidic linkages that can be formed, a diverse repertoire of glycan linkages is nevertheless constructed. The resulting glycome of mammals has characteristic features that structurally differentiate it from the glycomes of prokaryotes, early eukaryotes, invertebrates, and even some other vertebrates. We are studying the outcomes of heritable and acquired changes in the mammalian glycome that expose cryptic and phylogenically more primitive glycan linkages. These abnormal glycans are detected by endogenous lectin receptors. Heritable, and possibly acquired, exposure of cryptic glycan linkages can promote chronic inflammatory conditions that evolve into autoimmune disease by a novel mechanism in which pathogenesis is independent of the adaptive immune system. This mechanism of autoimmune disease onset cannot be easily detected at present in clinical settings as diagnostics are still lacking, yet this mechanism may play a significant role in the pathophysiology of common grievous diseases that include inflammatory, autoimmune, and degenerative syndromes that are of mysterious origin and which continue to escalate in the human population. We have further found that pathogen-

induced glycan re-modeling in mammals can also result in the exposure of phylogenically-primitive glycan linkages that are then recognized by phagocytic lectin receptors. In this case, lectin-ligand binding results in receptor-mediated endocytosis and clearance of deleterious glycoproteins from blood circulation, reflecting a protective mechanism that lessens the severity and improves the outcome of systemic infection.

Carbohydrates and Disease II (Monday)

031: Novel therapeutic glycoenzyme resources for lysosomal storage diseases

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Novel resources producing recombinant human lysosomal enzymes for enzyme replacement therapy (ERT) for lysosomal storage diseases (LSDs) have been developed. A methylotrophic yeast, *Ogataea minuta*, strain (Om4) has been established, in which not only *och1* (encoding α-mannosyltransferase) is deleted but also the introduced OmMNN4 (encoding a positive regulator of MNN6 gene product, a phosphomannosyltransferase) gene is overexpressed. A recombinant human β-hexosaminidase A (Om4HexA) produced by an Om4 strain co-expressing HEXA and HEXB genes, encoding α- and β-subunits of HexA, respectively, and then treated with bacterial α-mannosidase, contained N-glycans carrying high contents of terminal mannose 6-phosphate (M6P) residues. Replacement effects of the Om4HexA intracerebroventricularly administered to Sandhoff disease model mouse (*HEXB*-/- mouse) were demonstrated due to uptake via neural cell surface cation-independent M6P receptor (CI-M6PR). Therapeutic effects dependent on the M6P content were also observed on improvement of motor dysfunction as well as prolongation of the life span. A transgenic silkworm strain (Tg-CTSA) overexpressing the human CTSA (lysosomal protective protein/cathepsin A, CathA) in the middle silk glands was also established. The purified CathA carried human-like high mannose type and pauchi-mannose type N-glycans but lacked the core fucose residues. The conjugates composed of the CathA and cell-penetrating peptide (R8) were taken up by the fibroblasts derived from a patient with CathA deficiency, galactosialidosis (GS), and restored not only the CathA but also neuraminidase 1 (NEU1) and β-galactosidase (GLB), and reduced the accumulated sialyloligosaccharides in lysosomes. Glycoenzymes derived from silk glands have therapeutic potential to improve the symptoms and quality of life of the LSDs patients. This study was supported by NIBIO and Agri-health Translational Research Project.

032: The GD2 ganglioside induces a proliferative phenotype in MDA-MB-231 breast cancer cells via the constitutive activation of the receptor tyrosine-kinase c-Met

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Normal human tissues mainly express a-series gangliosides whereas complex gangliosides from b- and c-series are essentially found in developing tissues, during embryogenesis and restricted to the nervous system in healthy adults. In parallel, the expression of di- and trisialogangliosides increases in several pathological conditions including cancers. G_{D3}, G_{D2} and G_{T3} are considered as oncofetal markers in neuroectoderm-derived tumours such as melanoma and neuroblastoma, where they play a key role in tumour progression by mediating cell proliferation, migration, adhesion and angiogenesis. In breast cancer, G_{D3} is over-expressed in about 50 % of invasive ductal carcinoma and the G_{D3} synthase gene (ST8SIA1) displayed higher expression among estrogen receptor negative breast cancer tumours, associated with a decreased free survival of patients. However, no relationship between ganglioside expression and breast cancer development and aggressiveness has been reported. In order to determine the effect of complex gangliosides on breast cancer development, we have established a cellular model deriving from MDA-MB-231 breast cancer cells expressing the G_{D3} synthase, the key enzyme controlling b- and c-series gangliosides biosynthesis. The expression of G_{D3} synthase induces the accumulation of b- and c-series gangliosides (mainly G_{D2}) at the cell surface together with the acquisition of a proliferative phenotype in absence of serum or exogenous growth factors. G_{D3} synthase expression also induces an increased tumour growth of MDA-MB-231 cells in severe combined

immunodeficiency (SCID) mice. The analysis of tyrosine kinase receptors phosphorylation shows a specific and constitutive activation of c-Met receptor in G_{D3} synthase positive MDA-MB-231 cells and subsequent activation of Erk/MAPK and PI3K/Akt transduction pathways. Moreover, specific inhibitors of c-Met phosphorylation or c-Met siRNA reverse the proliferative phenotype. Finally, silencing of the G_{D2} synthase (β4GalNAc T1) efficiently reduces the proliferative phenotype due to the strong decrease of c-Met phosphorylation. Altogether, these results clearly demonstrate the involvement of the disialoganglioside G_{D2} in MDA-MB-231 cell proliferation via the constitutive activation of c-Met.

033: Identification and Visualisation of Glycated Proteins using Phenylboronate Acrylamide Gel Electrophoresis

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Background: During the post-translational modification of glycosylation, carbohydrates attach to proteins enabling them to maintain their function and correct three-dimensional structure. However, the undesired non-enzymatic process of glycation leads to formation of advanced glycation end-products (AGEs), which have implications in age-related chronic diseases such as diabetes, Alzheimer's disease, cancer and autoimmune diseases.

Results: We have developed a simple and cost-effective method that can separate and reliably detect glycated proteins using polyacrylamide gel electrophoresis (PAGE). Whilst PAGE cannot distinguish between glycated and unglycated proteins, by incorporating a specialised carbohydrate affinity ligand, methacrylamido phenylboronic acid (MPBA), we have now been able to specifically detect and separate glycated proteins (1). This novel electrophoresis technique, first developed to improve the separation of saccharides (2), not only differentiates between various carbohydrate-protein adducts in complex samples, but also discriminates early from late glycation states of proteins. In addition, we have now developed a new visualisation technique, using fluorescent boronic acids, to selectively label glycation products, enabling the identification of glycated proteins in PAGE before Coomassie or silver staining. This method has the potential added utility of acting as a diagnostic tool for age-related chronic diseases and to monitor disease progression.

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034: Roles of Mannose 6-Phosphorylation in Protein Trafficking and Cell Function Revealed by Mutant Mice (Keynote)

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More than 50 soluble acid hydrolases (e.g. glycosidases, proteases, lipases, phosphatases, nucleases, sulfatases) are involved in the degradation of cellular and extracellular macromolecules and inactivation of pathogenic organisms. The turnover of lysosomal constituents require a continous replacement with newly synthesized lysosomal proteins that have to be transported from the endoplasmic reticulum via the Golgi apparatus and endosomes to lysosomes. The majority of soluble lysosomal enzymes are modified with mannose 6-phosphate (Man6P) residues both in the α-1,3 and α-1,6 branches of their high mannose-type oligosaccharides. Man6P residues function as recognition signal for specific receptors in the Golgi complex and ensuing transport of the enzymes to lysosomes. The key enzyme in the two-step formation of Man6P residues is the hexameric GlcNAc-1-phosphotransferase complex ($\alpha_2\beta_2\gamma_2$) transferring GlcNAc-1-phosphate from UDP-GlcNAc to select C6 hydroxylgroups of mannoses. Mutations in the genes encoding GlcNAc-1-phosphotransferase subunits result in two human diseases, mucolipidosis II (ML II) and III. Biochemically these diseases are characterized by hypersecretion and intracellular deficiency of multiple lysosomal enzymes and the accumulation of nondegraded material in lysosomes. We generated a novel mouse model of MLII introducing a mutation detected in ML II patients that is associated with a complete loss of GlcNAc-1-phosphotransferase activity. The mutant ML II mice resemble the symptoms of human disease and show decreased viability, reduced body weight and body length, and severe skeletal abnormalities with shortened bones, spine deformity and facial dysmorphism. Histomorphometric analyses demonstrate a reduced bone volume per tissue volume accompanied by a reduced number of osteoblasts and an increased number of osteoclasts resulting in osteopenia. In the brain of the mutant mice atrophy and degeneration of neuronal cells was accompanied by microglial activation and the presence of storage vacuoles. The accumulation of nondegraded material such as GM2 and GM3 gangliosides, bis(monoacylglycero)phosphate (BMP), cholesterol and fucose-containing oligosaccharides arises from the loss of

specific lysosomal proteins that are strictly depend on Man6P for their transport to lysosomes. Thus, the transport of lysosomal enzymes along both the secretory and endocytic pathway was strongly altered in an enzyme-dependent manner in cultured fibroblasts or osteoblasts of mutant mice. The data indicate that the inability to form Man6P residues results in lysosomal dysfunction, accumulation of variable storage material, and impaired intracellular protein transport that subsequently lead to early cell death and organ failure.

Carbohydrates and Disease III (Monday)

035: Bioengineering of α2-8 polysialic acid-expressing cancer cells with N-propionyl mannosamine augments their cytotoxicity to human anti-N-propionylated polysialic acid antisera

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Mouse leukemic cancer cells can be targeted for immunotherapy by incubating them with N-propionyl mannosamine, an unnatural precursor of sialic acid biosynthesis, followed by subsequent treatment of the cells with mouse N-propionyl α2-8 polysialic acid-antibodies in the presence of complement [1]. Recently it was shown that an N-propionylated α2-8 polysialic acid–KLH conjugate vaccine was able to induce in humans, in addition to homologous N-propionylated α2-8 polysialic acid-specific antibodies, large quantities of potentially protective antibodies that cross-react with native α2-8 polysialic acid. In the current study the homologous antibody population was studied to evaluate its cytotoxic potential using α2-8 polysialic acid-expressing mouse cancer cells (RMA-s), both before and after their incubation with N-propionyl mannosamine. Following incubation, the RMA-s cells were shown to contain both N- propionyl- and residual N-acetyl α2-8 polysialic acid epitopes. However despite the presence of antibodies specific for both epitopes in the conjugate antisera, it was demonstrated by direct cytotoxic assays, and corresponding inhibition of cytotoxicity, that only antibodies specific for N-propionylated polysialic acid epitopes were able to promote strong cytotoxic activity.

[1] T. Liu, Z. Guo, Q. Yang, S. Sad, H. J. Jennings, J. Biol. Chem., 275, 32832 (2000)

036: Hypoxia-Induced Sialyl-Tn Antigen Expression Facilitates Tumor Metastasis

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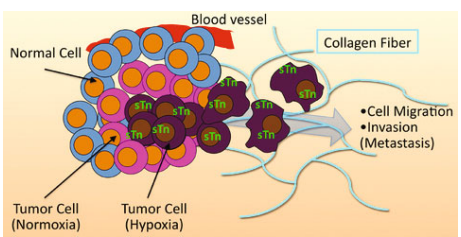
Sialyl-Tn (sTn) antigen is a tumor marker, the expression of which is well correlated with poor prognosis, although its biological function is not understood. sTn antigen is a carbohydrate epitope specifically synthesized by ST6GalNAc-I and is exclusively expressed in tumor cells. In the present study, we investigated the cellular physiological relevance of sTn antigen synthesis to tumor metastasis by characterizing the cellular architecture and motility and in vivo tumor development under hypoxic conditions. We have revealed that the ST6GalNAc-I promoter has two hypoxia response elements, which hypoxia inducible factor-1 binds and transactivates the promoter under hypoxic conditions. The hypoxia-induced ST6GalNAc-I expression resulted in the substantial expression of sTn antigen on a variety of plasmalemmal proteins and induced cellular morphological changes in vitro. The studies on human clinical lung cancer and mouse tumor xenograft revealed that sTn antigen expressing cells are appeared in the hypoxic regions in tumor. We engineered ST6GalNAc-I overexpressing cells using human non-small-cell lung carcinoma cells to elucidate cellular function of sTn antigen. The ST6GalNAc-I expression led to sTn antigen formation on a variety of proteins that was coincident with the morphological changes and the enhanced cellular motility that resemble the alterations in hypoxia-exposed cells. The sTn antigen expressing cells showed the enhanced focal adhesion complex formation that was coincident with the activation of focal adhesion kinase. In consistent with the facilitated formation of focal adhesion complex, the collagen receptor, α2β1 integrin, carried sTn antigen. The sTn antigen expressing cells exhibited enhanced collagen bindings that facilitated the collagen-dependent cell migration. The mouse tumor xenograft indicated that the sTn expressing cells deposited along with collagen fibers. We, furthermore, demonstrated that the sTn antigen expression significantly facilitated tumor intravasation. These findings indicate that hypoxia-induced sTn antigen expression on α2β1 integrin confers collagen-dependent cell migration that could contribute to promote tumor metastasis and progression.



037: Could Nanodiamonds be a Girl's Best Friend?

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The cause of human urinary tract infections (UTIs) are, in more than 75% of cases, due to the uropathogenic *Escherichia coli* (*E. coli*). Women are most susceptible to UTIs and effective drugs to counter persistent infections are currently lacking.1 E. coli are characterised by hair-like organelles constituted of several pilin subunits, including FimH. The latter is a lectin that binds mannopyrannose moieties. The multiple expression in *E. coli* of FimH-covered fibrillium accounts for the preferred recognition and adherence of these bacteria to mannose-terminating N-glycans in their host. Consequently the design of bacterial FimH agonists able to block bacterial adhesion and invasion in vivo has been identified as a therapeutic strategy and hotly pursued in recent years.2 However, the design of mimetics that can compete effectively with the multiply presented copies of FimH on the *E. coli* cell envelope has turned out not to be trivial. We wish in this paper to present the fabrication of nanodiamonds (NDs) covered with various sugars. These multivalent glyco-NDs represent to our knowledge the first of their kind to be reported. The ability of these novel glyco-NDs to inhibit various lectin-mediated phenomenons will also be presented. The majority of this new data will focus on the ability of glyco-NDs to interfere with the adherence of *E. coli* to various surfaces including those of yeast and human bladder cells. The effect on these novel glyco-NDs on the ability of *E. coli* to form biofilms will also be touched upon. Furthermore, these data will be compared with those data generated with other multivalent mannosylated structures.

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038: Defects in the biosynthesis of nucleotide-sugars and their transport into the Golgi (Keynote)

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'Congenital Disorders of Glycosylation (CDG)' comprise a rapidly growing group of inherited multisystemic human diseases caused by defects in glycoprotein biosynthesis that are evoked by mutations in glycosyltransferases and glycoprotein biosynthesis-associated proteins but also in proteins involved in the synthesis of nucleotide-activated monosaccharides and their transport into the Golgi.

In the first part, this presentation will give an overview on human defects in the biosynthesis of nucleotide-activated monosaccharides with a main focus on CDG-Ia, which is caused by deficiency of phosphomannomutase 2 (PMM2) and ends up in a severely reduced GDP-mannose level. The clinical phenotype of CDG-Ia is characterized by psychomotor and mental retardation, hepatopathy and blood clotting problems. Depending on the residual Pmm2 activity, CDG-Ia knock-out and hypomorphic mouse models present with a broad spectrum of phenotypes ranging from embryonic death to normal viability. In case of hypomorphic mice with a residual enzyme activity comparable to CDG-Ia patients, lethality in the mid-embryonic stage was accompanied by severe degradation of embryonic tissues and haemorrhage within the placenta. The growth of these embryos up to adulthood could be achieved by oral supplementation of the dams with D-mannose before and during pregnancy underlining the importance of correct glycosylation within a highly vulnerable period of embryonic development.

In the second part, human deficiencies in the transport of nucleotide-activated monosaccharides into the Golgi apparatus will be presented with a focus on CDG-IIc (Golgi GDP-fucose transporter deficiency) that causes severe recurrent infections with persistent leukocytosis, dysmorphism, growth and mental retardation. Knockout of the Golgi GDP-fucose transporter in mice leads to severely reduced protein fucosylation ending up in growth retardation and high postnatal mortality. Leukocyte rolling and lymphocyte homing are reduced, lung abnormalities might be due to defective growth factor signalling and severe behaviour abnormalities indicate the importance of correctly fucosylated glycoproteins in the brain.

Physiology and Signalling I (Tuesday)

039: Environmental control of *Dictyostelium* development is mediated via prolyl hydroxylation and glycosylation of the E3(SCF)ubiquitin ligase subunit Skp1 (Keynote)

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In the social soil amoeba *Dictyostelium discoideum*, the $E3^{SCF}$ubiquitin-ligase subunit Skp1 is modified at a specific proline residue by a novel pentasaccharide. This modification is assembled by an HIFα-like prolyl 4-hydroxylase (P4H1) and a novel cytoplasmic glycosylation pathway that appears to be conserved in many other protists including the agent for human toxoplasmosis, *Toxoplasma gondii*. Genetic perturbations of Dictyostelium P4H1 affect specific developmental checkpoints, including culmination and sporulation, in a way that suggests this enzyme is an oxygen-sensor as implied in animals. Analysis of the glycosyltransferase genes, whose actions depend on P4H1, show that their activities modulate P4H1 signaling suggestive of hierarchical control of development via successive sugar additions. Manipulations of Skp1 expression levels have inverse effects on oxygen-dependence. Together with evidence for genetic interactions between Skp1 and the modification genes, effects of proline mutations, and biochemical findings that Skp1 is the only substrate of these enzymes, Skp1 is strongly implicated as the target of P4H1-signaling during development. Whereas oxygen-dependent hydroxylation of the animal transcription factor HIFα triggers its polyubiquitination and degradation, P4H1 does not affect Skp1 stability. However, the consequence of Skp1 hydroxylation/glycosylation on cellular protein degradation may be similar if $E3^{SCF}$ubiquitin-ligase assembly or activity is affected. Assays indicate that the enzymes recognize highly conserved features of Skp1 that are blocked by partial SCF assembly, potentially restricting effects of environmental parameters to nascently synthesized Skp1. Recent evidence that Skp1 from Toxoplasma is similarly modified in a regulated manner suggests that oxygen-regulation of SCF Ub-ligases may be a general process that predates the association with HIFα and transcription that occurs in metazoa.

040: A glycosphingolipid/caveolin-1 signaling complex inhibits the motility of human ovarian carcinoma cells

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The genetic (stable overexpression sialyltransferase I - SAT-1 or GM3 synthase) or pharmacological (selective pressure by N-(4-hydroxyphenyl)retinamide)) manipulation of A2780 human ovarian cancer cells allowed us to obtain clones characterized by higher GM3 synthase activity respect to wild type cells. High GM3 synthase expression resulted in 1) elevated ganglioside levels, 2) reduced in vitro cell motility and 3) enhanced expression of the membrane adaptor protein caveolin-1. The motility of wild type, low GM3 synthase-expressing A2780 cells was reduced in the presence of exogenous gangliosides and by treatment with Brefeldin A (able to increase the cellular ganglioside levels). Treatment of A2780 cells with exogenous gangliosides only slightly increased the expression of caveolin-1, on the other hand it markedly increased the phosphorylation of caveolin-1 at tyrosine 14. In high GM3 synthase-expressing clones, both treatment with the glucosylceramide synthase inhibitor D-PDMP and transient silencing of caveolin-1 by siRNA were able to strongly increase cell motility. The non-receptor tyrosine kinase c-Src plays a crucial role in controlling the motility of these cells: 1) the motility of low GM3 synthase-expressing cells was reduced in the presence of a Src inhibitor; 2) c-Src was less active in high GM3 synthase-expressing clones; 3) D-PDMP treatment of cells high GM3 synthase-expressing cells led to c-Src activation. In high GM3 synthase-expressing cells, caveolin-1 directly interacted with GM3 ganglioside and was associated with sphingolipids, integrin receptor subunits, p130Cas and c-Src forming a non-caveolar signaling complex, insoluble in both Triton X-100 and Brij 98. These data suggest a novel role for gangliosides in regulating tumor cell motility, by affecting the organization of a signaling complex organized by caveolin-1, responsible for Src inactivation downstream to integrin receptors, and imply that GM3 synthase is a key target for the regulation of cell motility in human ovarian carcinoma.

041: β1,4-galactosyltransferase V regulates the endothelial differentiation and tumorigenicity of glioma-initiating cells

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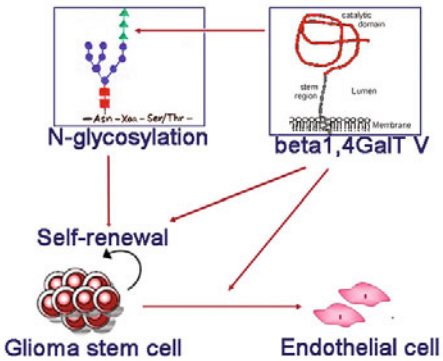
Glioma-initiating cells (GICs) play pivotal roles in glioma initiation, growth and recurrence, and therefore, their elimination is an essential factor for the development of efficient therapeutic strategies. However, the regulatory pathways which are essential for sustaining stemness and tumorigenicity of GICs are largely unknown. Cell surface N-linked oligosaccharides play functional roles in determining cell fate, such as self-renewal, proliferation and differentiation. Its altered expression is associated with glioma malignancy. Previously, we have reported that β1,4-galactosyltransferase V (β1,4GalT V) which effectively galactosylates the GlcNAcβ1→6Man arm of the highly branched N-glycans functions as a glioma growth activator. In this study, decreasing the expression of β1,4GalT V by RNA interference in glioma cells inhibited the ability of tumor formation and angiogenesis in vivo. Furthermore, knockdown of β1,4GalT V depleted CD133/nestin-positive cells in glioma cell xenografts and inhibited the self-renewal capacity and the tumorigenic potential of glioma-initiating cells isolated from glioma xenografts and patient tissues. Consistent with this,

N-glycan synthesis inhibitors tunicamycin and swainsonine reduced the self-renewal capacity and the tumorigenic potential of glioma-initiating cells. More interestingly, β1,4GalT V down-regulation inhibited endothelial differentiation of GICs. Collectively, our results reveal the critical role of β1,4GalT V in controlling the endothelial differentiation and tumorigenicity of GICs, and manipulating β1,4GalT V expression may have therapeutic potential for the treatment of malignant glioma.



042: Role of nucleocytoplasmic lectins in plant cell signaling

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During the last decade it was shown that plants also synthesize lectins in minute amounts in response to some specific stress factors and changing environmental conditions. Further localization studies have shown that this group of lectins locates to the nuclear and/or cytoplasmic compartment of the plant cell. Since the expression of several nucleocytoplasmic lectins was shown to be inducible by (a)biotic stress factors the hypothesis was put forward that these lectins are involved in stress signaling (Lannoo and Van Damme, 2010). In 2002 the *Nicotiana tabacum* agglutinin (Nictaba) was purified from tobacco leaves (*Nicotiana tabacum* cv Samsun NN) (Chen et al. 2002). The lectin is not expressed under normal physiological conditions, but accumulates in leaf parenchyma cells after treatment with certain jasmonates. Hitherto the molecular function of Nictaba remains unknown. We used a proteomics approach to isolate and identify Nictaba-interacting proteins in the nucleus and the cytoplasm of *Nicotiana tabacum* cv Xanthi cells using lectin affinity chromatography as well as pull down assays (Schouppe et al. 2011). Both approaches revealed that Nictaba primarily associates with core histone proteins. Binding of Nictaba to these nucleosomal proteins was confirmed in an affinity chromatography experiment with a calf thymus histone preparation. Proteins were eluted from the column with GlcNAc monomers. The O-GlcNAc modification of histone proteins was also proven by MS analysis. Our data suggest that Nictaba binds to several O-GlcNAcylated plant proteins in the nuclear compartment, in particular histone proteins. These results are in agreement with previous observations showing the specific interaction of Nictaba with GlcNAc oligomers. Future studies will concentrate on the ultrastructural co-localization of Nictaba and histone proteins in the plant cell, and the functional implications resulting from this interaction. Chen Y, et al. (2002) FASEB J 16: 905-907; Lannoo N and Van Damme EJM (2010) Biochem Biophys Acta 1800: 190-201; Schouppe D, et al. (2011) Plant Physiol 155, 1091-1102

Physiology and Signalling II (Tuesday)

043: Role of myeloid siglecs in bridging innate and adaptive immunity (Keynote)

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The siglecs are a family of 14 sialic acid binding Ig-related membrane proteins mostly expressed on different cells of the immune system with the exception of T cells in humans and mice. The prototypic siglec, sialoadhesin (siglec-1), is a macrophage-restricted cell adhesion molecule which is found on major subsets of tissue macrophages in secondary lymphoid organs and is upregulated during inflammatory responses. It has broad specificity for sialylated glycans in glycoproteins and glycolipids, but depends on clustering of both receptors and ligands to mediate high avidity binding. Siglec-E is a myeloid restricted receptor in the mouse that is homologous to human proteins siglecs-7 and -9 which also has broad sialic acid binding activity. To investigate the roles of sialoadhesin and siglec-E in cellular interactions with T cells, we have analysed changes in ligand expression following in vitro and in vivo T cell activation. Our results indicate selective upregulation of ligands for each receptor that are regulated via controlled expression of glycan modifying enzymes. In the case of sialoadhesin, ligand upregulation on subsets of T cells correlates with T cell activation and can result in altered T cell survival following receptor engagement. Our findings indicate a close interplay between siglec expression on myeloid cells of the innate immune system and siglec ligand modulation on T cells that is likely to be important in fine-tuning adaptive immune responses, both to pathogens and in autoimmune disease.

044: Shiga toxin receptors in leukocyte-derived cell lines: subcellular distribution of globo-series neutral glycosphingolipids and expression analysis of related glycosyltransferases

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Shiga toxins (Stxs) are AB5 toxins composed of a single A-subunit and 5 identical B-subunits. The catalytic A-subunit has rRNA N-glycosidase activity and inhibits eukaryotic protein biosynthesis. The pentameric B-subunit binds to the high and less effective neutral glycosphingolipid (GSL) receptors globotriaosylceramide (Gb3Cer/CD77) and globotetraosylceramide (Gb4Cer), respectively. Leukocyte-derived cell lines of the myeloid and lymphoid lineage are known to express Stx receptors. In a recent study we identified Gb3Cer (d18:1, C24:1/C24:0) and Gb3Cer (d18:1, C16:0) as the prevalent Stx-receptors in a B cell-derived cell line and in addition Gb4Cer (d18:1, C24:1/C24:0) and Gb4Cer (d18:1, C16:0) in a monocytic cell line. Here we present novel data on the in-depth evaluation of subcellular distribution of Stx receptors and the sensitivity towards Stx in both cell types. Since organization of GSLs in lipid rafts is believed to be the prerequisite for effective internalization of Stxs, we characterized the microdomain-associated GSLs in lymphoid and myeloid cell lines by means of detergent resistant membranes (DRMs) obtained by sucrose density gradient ultracentrifugation. Stx receptors were found to predominantly distribute to DRMs revealing their association with and enrichment in lipid rafts. Furthermore, studies on the expression of glycosyltransferases β1,4GalT5, β1,4GalT6, α1,4GalT and β1,3GalNAcT involved in the biosynthesis of globo-series neutral GSLs employing real time PCR showed high compliance with structural GSL analysis and results of the Stx-mediated cytotoxicity. Our data on the expression of Gb3Cer and Gb4Cer and their structural diversity in lymphoid and myeloid cell lines support the hypothesis that different lipoforms of Stx receptors might play a functional role in the molecular assembly of GSLs in membrane organization and cellular signaling in Stx-susceptible cells.

This work is supported by grants from the Deutsche Forschungsgemeinschaft (DFG)-funded International Graduate School (GRK 1409).

045: Withdrawn

046: Galectin-binding to specific glycoforms of serum glycoproteins: mechanisms of selectivity, functional consequences, and relationships to disease.

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Most serum proteins carry carbohydrate side chains, mainly as N-glycans. Changes in these glycans have long been observed in conjunction with disease such as inflammation and cancer, and in a few cases are used in routine diagnosis, but almost nothing is known on what biological roles these glycan changes have. We have now discovered that specific glycoforms of major serum proteins are bound by galectins, a family of β-galactoside binding proteins found in tissue cells.

Mechanisms: Using a newly developed fluorescence anisotropy assay, it can be demonstrated that the selective binding of galectins to glycoproteins involve more specific aspects of common N-glycans and neighboring proteins parts than previously assumed. This has been studied with asialofetuin and fetuin as a model glycoproteins, e.g. showing dramatically different binding of galectin-1 compared to galectin-3, and with haptoglobin and transferrin as relevant serum glycoproteins.

Functions: The binding of glycoproteins to galectins in tissue cells results in different intracellular sorting after endocytosis. This has been shown with uptake of transferrin in various cells and with uptake of haptoglobin-hemoglobin complexes in alternatively activated macrophages.

Relationships to disease: Increase or decrease of specific galectin-binding glycoforms in serum correlates in different ways with diseases. An increase in galectin-1 binding glycoforms and decrease in galectin-8 binding glycoforms correlates with breast cancer, whereas the reverse is true in IgA-nephritis. Galectin-8 is the only galectin binding IgA, and galectin-8 non-bound IgA correlates with IgA-nephritis.

O-GlcNAc: New Frontiers (Society for Glycobiology Guest Session; Tuesday)

047: GlycoEpigenetics: Is the ghost in your genes a sugar?

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The nutrient-sensing hexosamine signaling pathway terminating in O-GlcNAc cycling is emerging as a key epigenetic regulator of gene expression in mammals Mounting evidence suggests that O-GlcNAc cycling sits atop a robust regulatory network maintaining higher-order chromatin structure and epigenetic memory. O-GlcNAc cycling occurs on many cellular targets associated with signaling, morphogenesis and transcriptional regulation including nuclear pores, RNA polymerase II, Sin3A, and many transcription factors. In addition, it has now been added to the multifaceted 'histone-code'. In model systems ranging from *C. elegans* to man, O-GlcNAc cycling has been shown play an important role in modulating developmental plasticity. We have focused on the influence of O-GlcNAc cycling upon the molecular modifications of the RNA Polymerase II carboxyl-terminal tail. In *C. elegans*,

O-GlcNAc marks the promoters of over 800 developmental, metabolic, and stress-related genes. These O-GlcNAc marked genes show a 5' bias in the distribution of RNA Polymerase II (Pol II) suggestive of enhanced promoter-proximal pausing. In response to starvation or feeding, levels of O-GlcNAc at promoters remain nearly constant due to dynamic cycling mediated by the transferase OGT-1 and the O-GlcNAcase OGA-1.However, in viable mutants lacking either of these enzymes of O-GlcNAc metabolism, the nutrient-responsive O-GlcNAcylation of promoters is dramatically altered. Blocked O-GlcNAc cycling leads to a striking nutrient-dependent accumulation of O-GlcNAc on RNA Pol II. O-GlcNAc cycling mutants also show an exaggerated, nutrient-responsive redistribution of promoter-proximal RNA Pol II isoforms and extensive transcriptional deregulation. These studies in the nematode have been extended to the fly and vertebrate RNA Pol II CTD. Our findings suggest a complex interplay between the O-GlcNAc modification at promoters and the kinase-dependent 'CTD-code' regulating RNA Pol II dynamics. Nutrient-responsive O-GlcNAc cycling may buffer the transcriptional apparatus from dramatic swings in nutrient availability by sustaining a 5' pool of RNA Pol II poised for rapid reactivation to meet metabolic and developmental fate decisions.

048: Extensive Crosstalk Between O-GlcNAcylation & Phosphorylation: A New Paradigm for Nutrient Regulation of Signaling, Transcription and Mechanisms Underlying Chronic Disease.

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O-GlcNAcylation of nuclear and cytoplasmic proteins serves as a nutrient/stress sensor to regulate signaling, transcription and cellular metabolism. Recent phospho- and glycomic approaches have shown that an increase in global O-GlcNAcylation affects phospho-site occupancy at nearly every actively cycling site. A chemico-enzymatic photochemical enrichment method, combined with ETD-mass spectrometry allows detection of O-GlcNAc site occupancy at a level of sensitivity comparable to that possible for phosphorylation. These analyses show that crosstalk between site-specific phosphorylation and O-GlcNAcylation is extensive. Several kinases are both modified and regulated by O-GlcNAcylation. The major sensor of cellular energy state, AMPK is O-GlcNAcylated. AMPK and O-GlcNAc transferase share many substrates and the two systems directly interact. Major signaling cascades (e.g. CDK1, aurora kinase, polo kinase) that regulate cell division are strikingly affected by a small change in O-GlcNAcylation. O-GlcNAc is part of the histone code, but many of the O-GlcNAc residues are in sites interacting with DNA in the nucleosome. Multiple core ribosome proteins are modified by O-GlcNAc, which plays a role in ribosome biogenesis and assembly. Thus, O-GlcNAcylation modulates many signaling cascades and phosphate-mediated molecular switches to 'tune' them to be highly responsive to nutrients and stress.

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049: Spatiotemporal Organization of OGT and O-GlcNAcylated Proteins in Lipid Rafts for Effective Insulin Signalling Pathway

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The attachment of O-linked beta-N-acetylglucosamine (O-GlcNAc) to proteins is an abundant and reversible modification that is involved in many cellular processes including transcription, translation, cell proliferation, apoptosis and signal transduction. Two enzymes are responsible for the addition and the removal of the O-GlcNAc modification: the uridine diphospho-N-acetylglucosamine:polypeptide β-*N*-acetylglucosaminyltransferase (O-GlcNAc transferase or OGT) and the *N*-acetyl-β-glucosaminidase (O-GlcNAcase or OGA), respectively. Although the functions of O-GlcNAcylation have been studied at different levels of cell regulation, there was none study until now referring its potential location at the level of the lipid rafts. Nevertheless, it has recently been shown that elevated levels of O-GlcNAcylation are actively involved in the phenomenon of insulin resistance and a significant number of publications also reports the critical role of lipid microdomains in this phenomenon of resistance. Little while ago, we know that after stimulation with insulin, the OGT is recruited to the plasma membrane through its PPO domain (PIP-binding activity of OGT), and that this relocation inactivates the PI3-kinase/Akt signaling pathway by exerting an antagonistic effect on its phosphorylation. These three observations suggest that OGT through its interaction with the plasma membrane and more particularly with the lipid microdomains may play an essential role in the signal transmission. Our experiments show for the first time that OGT is expressed in the lipid rafts, In addition to OGT we also found previously described O-GlcNAcylated proteins associate with the lipid microdomains e.g. the insulin receptor chain B, HSP70 and OGT itself. The recruitment of these elements is controlled by the insulin: we observed a progressive association of proteins O-GlcNAc to the lipid microdomains after the stimulation for this hormone. Our results show that in response to the stimulation, that is the binding of the insulin on its receptor, the OGT is recruited to lipid rafts, suggesting a downregulation of the PI3-Kinase/Akt pathway by

O-GlcNAcylation at the level of the lipid microdomains. This study proposes a direct relationship between the O-GlcNAcylation of the lipids rafts-associated proteins and the regulation of the insulin signaling pathway. Faults of OGT spatiotemporal organization in the lipid rafts might have a potential implication in the etiology of the type 2 diabetes.

050: Roles of O-GlcNAc modification on Snail in Epithelial-Mesenchymal Transition

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The protein Snail plays a key role in epithelial-mesenchymal transition (EMT) by direct repression of E-cadherin transcription. Therefore, regulation of Snail expression level in epithelial tumor cells is important not only for maintaining of epithelial homeostasis, but also for invasion and metastasis of cancer cells by the EMT program. Series of Ser imbedded in Snail are phosphorylated by GSK3 and Snail expression is dynamically regulated by Wnt signaling together with β-catenin while driving a Snail-dependent EMT program. Glucose flux through the hexosamine biosynthetic pathway (HBP) can be used for the source of O-linked β-N-acetylglucosamine (O-GlcNAc) modification on Ser and Thr residues of various nucleocytoplasmic proteins. In this study, we demonstrate that Ser 112 of Snail is O-GlcNAcylated and this adjacent-site occupancy inhibits phosphorylation by GSK-3, resulting in increased Snail stability and attenuation of E-cadherin proximal promoter activity and transcription level. Furthermore, Overexpression of OGT induces in vivo invasion program of epithelial cancer cells by Snail-dependent manner. Taken together, our results indicate dynamic interplay between O-GlcNAcylation and GSK-3 phosphorylation of Snail, and our observations may provide the molecular insight of pathogenic and prognostic correlation between cancer progression and hyperglycemic condition of diabetes.

051: Dynamic O-GlcNAc Glycosylation Regulates Neuronal Gene Expression and Memory Storage

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O-Linked-β-N-acetylglucosamine (O-GlcNAc) glycosylation is a dynamic, intracellular modification that shares features with protein phosphorylation. We have developed chemical approaches to accelerate the discovery and study of O-GlcNAc glycosylated proteins. Here, we will discuss new developments in these approaches and their application to understanding the signaling pathways that regulate O-GlcNAc glycosylation in neurons. In addition, we have discovered that cAMP-responsive element binding protein (CREB) is O-GlcNAc modified in vivo, and we will describe the functional implications of CREB glycosylation for gene expression, axonal and dendritic growth, and long-term memory storage.

052: Protein O-GlcNAcylation: A key mediator of cardiomyocyte function and survival

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The modification of Ser/Thr residues of nucleocytoplasmic proteins by O-linked β-N-acetyl-glucosamine (O-GlcNAc) is rapidly emerging as a key regulator of numerous biological processes. Although increased O-GlcNAcylation has been implicated in the adverse effects of diabetes on the heart, we have demonstrated that acute activation of O-GlcNAcylation either by increasing O-GlcNAc synthesis or inhibiting its degradation, is remarkably cardioprotective. Of particular importance, in models of myocardial infarction or hemorrhagic shock, administration of O-GlcNAcase inhibitors at the time of reperfusion/resuscitation significantly improves outcomes as indicated by reduced tissue injury and improved survival. One protective mechanism is decreased pro-inflammatory responses, characteristic of tissue injury, which is due to O-GlcNAc meditated attenuation of NF-κB activation. This is in contrast to studies demonstrating that the pro-inflammatory effects of hyperglycemia are due to increased O-GlcNAcylation of NF-κB. However, despite considerable evidence demonstrating the importance of protein O-GlcNAcylation in mediating cardiomyocyte stress responses, the mechanisms regulating cardiomyocyte O-GlcNAc levels are poorly understood. While cellular O-GlcNAc levels rapidly increase in response to stress, we have found that oxidative stress such as that, which occurs during reperfusion or resuscitation, leads to a marked loss of overall O-GlcNAc levels, which is associated with reduced levels of OGT protein. Paradoxically, removal of glucose, the primary substrate for O-GlcNAc synthesis is a potent stimulus for increasing cardiomyocyte O-GlcNAc levels, and this appears to be mediated by decreased flux through the hexosamine biosynthesis pathway. Thus, protein O-GlcNAcylation clearly plays a key role in mediating cardiomyocyte function and survival and had as implications for our understanding of cellular mechanisms contributing to the adverse effects of ischemia, hypertrophy, diabetes and aging on the heart.

053: Molecular mechanisms of O-GlcNAc signalling

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Many proteins in the eukaryotic cell are modified by O-linked N-acetylglucosamine (O-GlcNAc) on serines and threonines. O-GlcNAcylation has been shown to be important for regulation of the cell cycle, DNA transcription and translation, insulin sensitivity and protein degradation. Misregulation of O-GlcNAcylation is associated with diabetes and Alzheimer's disease. Two enzymes are involved in the dynamic cycling of this posttranslational modification, the O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). It has been demonstrated that this posttranslational modification occurs on some serines/threonines that are also known phosphorylation sites for a number of key kinases, giving rise to the "yin-yang" theory, that proposes that O-GlcNAcylation is a means of regulating protein phosphorylation. Work in my group is aimed at studying this mechanism. We are studying the structures of OGA and OGT to gain insight into substrate recognition, and we have developed highly potent and selective inhibitors to study O-GlcNAcylation in live cells. Using these tools were are currently studying the role of O-GlcNAc in signalling pathways involved in diabetes, cancer and Alzheimer's disease.

Infection & Immunity I (Tuesday)

054: Sialic acid mediated interaction between *Pseudomonas aeruginosa* and neutrophil through siglec-9 reduced innate immune response

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Pseudomonas aeruginosa (PA) is an environmentally ubiquitous, extracellular, opportunistic pathogen that is associated with severe infections of immunocompromised host. Siglec-9 presents on the surface of neutrophil interact with sialic acid (Sia) on the neutrophil own surface by cis interaction and thereby reducing its own immunological activity (1-2). However, such interactions engage neutrophil with other host cell or a pathogen in trans interaction. PA acquired SA from the environment and utilized for their survival in host by reducing complement deposition on sialylated PA (3). This observation prompted us to investigate details mechanism of interaction of PA-Sia with human neutrophil and their role in innate immune response. Sia on PA showed significant binding with siglec-9 on neutrophil. Such interaction showed reduced neutrophil oxidative burst, release of granule proteases and extra cellular trap formation. Increased genetic expression of IL-10 along with enhanced intracellular and secreted IL-10 was also found. Taken together, these observations established the role of acquired sia on PA that can weaken neutrophils protective activity as supported by their enhanced CFU count i.e. survivality. Thus this is a unique mechanism for survival of PA by decreasing innate immunity of host through its Sia.

References: 1. Crocker et.al. (2007) Nat. Rev. Immunol. 7, 255 2. Carlin et.al (2009) Blood. 113, 3333 3. Khatua et.al (2010) FEBS Letters. 584, 555 The work is funded by CSIR, DBT and ICMR and BK is a SRF (CSIR)

055: Complement L-ficolin binds to surface glycans of HCV and HIV and reduces these viral infectivities, and functions as an antiviral opsonin

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L-ficolin is a recently identified complement lectin, which recognizes bacterial carbohydrates, thereby activate lectin complement pathway. However little is known about the role of L-ficolin in viral infections. This report shows that human L-ficolin specifically binds to surface glycans of HCV and HIV, and subsequently activates the lectin complement pathway and complement mediated cytolytic activity. Moreover, we found that L-ficolin could significantly block the infections of HCV and HIV to the target cells, and functioned as an antiviral opsonin in vitro. Furthermore, we found that serum levels of L-ficolin in patients of HCV and HIV were significantly increased. These new findings will contribute to the development of L-ficolin as a novel immunotherapy agent against the infections of these important human viruses.

056: Soluble lectins and superlectins from opportunistic bacteria

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Recent interest in bacterial lectins demonstrated their role in host recognition, biofilm formation, tissue adhesion and virulence. *Pseudomonas aeruginosa* and *Burkholderia* species are opportunistic pathogens responsible for lung infections that are life-threatening for cystic fibrosis patients and immuno-compromised individuals. Both bacteria contain several soluble lectins that demonstrate high affinity for diverse oligosaccharides that are present on human tissues. We used combined titration microcalorimetry, x-ray crystallography and molecular modeling approaches to decipher the thermodynamical and structural basis for high affinity binding of bacterial lectins to host carbohydrates. Four different groups of lectins or superlectins, constituted of several domains, have been characterized. Their role in the infection is not completely elucidated but the lectins are involved bacteria aggregation, biofilm formation, tissue recognition and triggering of inflammation process. The complete characterization of carbohydrate specificity, affinity and atomic details of interaction between some

bacterial lectins and their ligands allowed for the design and synthesis of high affinity glycomimetics and glycodendrimers that can act as antiadhesive compounds. The affinity of bacterial lectins for carbohydrate derivatives can also be used for diagnostic approaches by nanoelectronic detection of lectin-carbohydrate interactions using carbon nanotubes.

057: Essential protein O-glycosylation in *Bacteroides* species (Keynote)

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Bacteroides is a genus of bacteria that is predominant in the human colonic microbiota. Bacteroides species have a general *O*-glycosylation system that is notable in that glycans are added to serine and threonine residues contained within the three amino acid motif (D)(S/T)(A/L/V/I/MT) of extracytoplasmic proteins. We have found that there are likely hundreds of proteins that are glycosylated in *Bacteroides fragilis*, some of which are encoded by essential genes. We extended our initial analyses of the protein glycosylation system in *B. fragilis* to demonstrate that proteins of this organism are modified with two major types of glycans, a larger species specific glycan and a smaller glycan composed of two sugars. We previously demonstrated that mutants Dlfg and DgmdfclDfkp, which have a defect in protein glycosylation, have impaired in vitro growth. We show that proteins of these mutants are still glycosylated, but only with the small glycan. Therefore, the in vitro growth defect is not due to absence of protein glycosylation, but rather lack of glycosylation with the larger glycan. We found that phylogenetically diverse species within the phylum Bacteroidetes glycosylate proteins with a glycan that is immunologically similar to the small glycan of *B. fragilis* glycoproteins, and use the same three amino acid motif for glycosylation. The wide-spread conservation of this protein glycosylation system within the phylum suggests that this system of post-translational protein modification evolved early before the divergence of the three classes of bacteria within this phylum and has been maintained due to its physiologic importance to these bacteria.

Infection & Immunity II (Tuesday)

058: Altering our prejudgements: neutral, zwitterionic and negatively-charged N-glycans in parasites and other lower eukaryotes

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Traditionally, the N-glycans of 'simple' organisms were considered to be also 'simple', whereas negatively-charged glycans were generally considered to be only present in vertebrates. However, it has become recently obvious that the N-glycans of parasitic species as well as their non-parasitic 'cousins' display a degree of complexity and novelty which was previously overlooked. Naturally, advances in glycan analysis as well as changing paradigms mean that novel structures are now being discovered - partly we should talk of 'complicated' structures which do not fall into the classical 'complex' category. Furthermore, the virulence of parasitic organisms is in part based on their ability to hijack host glycan-based recognition systems and indeed skew the host immune system in a glycan-dependent manner. As part of recent and ongoing independent projects, we have examined the glycans of a number of species which are either parasitic (nematodes or trichomonads) or are models closely or distantly related to parasites (*Caenorhabditis elegans* and *Dictyostelium discoideum*) or are invertebrates which are models for a host-parasite interaction (the oyster *Crassostrea virginica*). In all these cases, either zwitterionic or negatively-charged N-glycans can be found as well as neutral structures, some of which are reminiscent of mammalian glycans or which share features with glycans with known immunogenic or immunomodulatory potential. The analysis of these glycans not only presents its own challenges, but may offer clues as to how parasites interact with their hosts.

059: A novel effector protein in the defence of mushrooms against predators and parasites

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We identified a novel lectin, termed CCL2, from the saprobic mushroom *Coprinopsis cinerea* by virtue of its binding to the plant glycoprotein horseradish peroxidase

(HRP). Like many fungal lectins, CCL2 is highly soluble, lacks a signal sequence for classical secretion and is specifically produced in the fruiting body. Homologous proteins are encoded in the genome of *C. cinerea* itself and in the genomes of several other mushrooms. In vitro binding analysis of recombinant CCL2 revealed a pronounced specificity and high affinity of the lectin for the trisaccharide GlcNAcβ1,4(Fucα1,3)GlcNAc found at the N-glycan cores of plants, insects and nematodes but not of fungi. NMR studies showed that the monomeric lectin adopts a beta-trefoil (R-type) fold and coordinates a single ligand molecule at a site different from the canonical carbohydrate-binding sites of R-type lectins. Toxicity bioassays with the model organisms *Drosophila melanogaster* and *Aedes aegypti* (insects), *Caenorhabditis elegans* (nematode) and *Acanthamoeba castellanii* (amoeba) revealed a strong toxicity of CCL2 towards *D. melanogaster* and *C. elegans*. Resistance of *C. elegans* mutants defective in the biosynthesis of the N-glycan α1,3-core fucosides confirmed that the nematotoxicity of CCL2 is dependent on the in vivo binding of this specific glycoepitope. Feeding C. elegans with a dTomato-CCL2 fusion protein showed that binding of CCL2 occurs at the intestinal epithelium of the nematode and leads to a disintegration of this tissue. Our results suggest the existence of a protein-mediated defense system of fungi against predators and parasites in which fruiting body lectins such as CCL2 act as effector proteins by binding to specific glycoepitopes in the target organisms.

060: Role of ergosterol dependent and glycosphingolipid-enriched detergent resistant membrane microdomains of *Histoplasma capsulatum* in macrophage infection.

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Aiming to analyze the organizational structure of detergent resistant membrane microdomains (DRMs) of yeast forms of *Histoplasma capsulatum* and the relationship of DRM components with fungal viability and their ability to infect mouse alveolar macrophage, we performed a series of experiments with methyl-beta-cyclodextrin (mßCD) which showed the presence of two types of DRMs: i) ergosterol-dependent DRMs and ii) glycosphingolipid enriched/dependent DRMs. About 40% of ergosterol and 25% of glycosphingolipids (GSLs) of *H. capsulatum* yeasts are present in membrane microdomain fractions resistant to detergent treatment (1% Brij 98) at 4°C. Specific proteins were also enriched in these DRMs, particularly: Pma1p, a fungal plasma membrane proton ATPase and microdomain protein marker; a (glyco)protein of 30kDa, able to bind to laminin; and a 50kDa protein recognized by mAb anti-α5-integrin. Removal of 85% of ergosterol of *H. capsulatum* by mßCD led to the displacement of Pma1p and the 30kDa (glyco)protein from DRM fractions to detergent soluble fractions in the sucrose gradient. mßCD treatment did not alter fungal viability but reduced 45% of the fungal ability to infect alveolar macrophages. Remarkably *H. capsulatum* treatment with mßCD did not displace GSLs or α5-integrin-like 50kDa protein from DRM fractions to detergent soluble fractions. Reinsertion of ergosterol into membranes of mßCD-treated yeasts restored their ability to infect alveolar macrophages whereas insertion of exogenous soluble cholesterol did not restore *H. capsulatum* infectivity. These data indicate the existence of two sub-populations of DRMs in *H. capsulatum*: type I DRMs with structural integrity depending specifically of ergosterol, possibly related to macrophage infectivity and type II DRMs possibly related to signaling processes and fungal development rich in integrin-like proteins and GSLs with the structures: Man*p*(α1—3)[Gal*f*(ß1—6)] Man*p*(α1—2)IPC, Man*p*(α1—3)Man*p*(α1—2)IPC and Glc(ß1—1)Cer. Studies to characterize the roles of ergosterol and glycosphingolipids in fungal infectivity and signaling processes may represent new therapeutic approaches to histoplasmosis. Supporter by FAPESP, CNPq and CAPES

061: Structural and functional insight into how *Plasmodium falciparum* infected erythrocytes adhere to Chondroitin Sulfate A in the human placenta (Keynote)

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The malaria parasite *Plasmodium falciparum* expresses a group of heterogeneous multi-domain proteins called *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) on the surface of infected erythrocytes, which mediate binding to receptors on the vascular lining. This interaction is essential for the parasite, but harmful to the human host. VAR2CSA, a member of the PfEMP1 family, sequestrates parasites to the placenta by binding to low-sulphated form of chondroitin sulphate A (CSA) found anchored to proteoglycans (CSPG) in the intervillous space of the placenta. To aid vaccine development, we compared low-resolution molecular structures of the CSA binding VAR2CSA and an ICAM binding PfEMP1. Binding affinity measurements on a range of VAR2CSA fragments were used to define the CSA binding region. The relevance of the findings was confirmed by showing that antibodies against this region block the receptor interaction of native VAR2CSA. The results indicate that VAR2CSA binding is placed in a

compact, multi-domain fold located in the N-terminal part of the molecule. By contrast the ICAM1 binding PfEMP1 has an elongated shape, which could support a simpler single domain-receptor interaction.

Infection & Immunity III (Tuesday)

062: Limiting the amounts of Dol-P-Man could be the answer in curing African sleeping sickness.

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Infectious diseases caused by parasitic protozoa constitute one of the greatest public health issues of humanity, infecting 15% of the global population with high morbidity and millions of fatalities annually. The neglected disease, African sleeping sickness caused by the protozoan parasite *Trypanosoma brucei* that is transmitted by the bite of the Tsetse fly, affects millions across sub-Saharan Africa, current drug treatments are woefully inadequate and new therapies are urgently required. Dolichol-phosphate-mannose (Dol-P-Man) is synthesised by the ER resident Dol-P-Man synthase (DPMS) utilising dolichol-phosphate and GDP-mannose. Dol-P-Man is a key intermediate in eukaryotic glycosylation pathways, being a mannose donor in both N-glycosylation and glycosylphosphatidylinositol (GPI) anchor biosynthesis. These pathways are of particular importance in the formation of the protective cell-surface coat of the bloodstream parasite *T. brucei*, namely the Variant Surface Glycoprotein (VSG). Recombinant expression of the full-length (membrane-bound) TbDPMS has allowed enzymatic assays to explore substrate/inhibitor specificity, as well as identification of active-site residues through mutagenesis. A conditional knockout of TbDPMS demonstrates the gene is essential, unsurprising as GPI biosynthesis has previously been genetically and chemically validated as a drug target in the bloodstream form of the parasite. However by using RNAi manipulations of TbDPMS, we are able to investigate the biochemical phenotype caused by a reduction of DPMS activity in bloodstream *T. brucei*. In particular, we assess the associated consequences to VSG glycosylation, addressing whether under limiting amounts of Dol-P-Man, the parasites sacrifice N-glycosylation or GPI anchor biosynthesis. The resulting modifications to the VSG are surprising and highlights the effort to which the parasite will go to maintain the integrity of their VSG coat. These findings allow us to propose a novel therapeutic stratergy in the fight against African sleeping sickness and other neglected third world diseases.

063: The envelope glycans of HIV are predominantly oligomannose regardless of production system or viral clade

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The human immunodeficiency virus (HIV) antigen, gp120, is covered by an extensive array of N-linked glycans. A key feature of gp120 glycan shield is an unusual high abundance of incompletely processed oligomannose-type glycans, which are thought to enhance infectivity by binding to DC-SIGN on peripheral dendritic cells. These unprocessed oligomannose glycans also form the basis of the epitope of 2G12, a mannose-specific broadly neutralizing antibody against HIV. Envelope glycans have become an attractive target for vaccine design, and we sought to investigate the glycosylation of gp120 as found on the virus. We firstly investigated different pseudoviral production systems in 293T cells. Envelope glycoprotein shed from pseudovirus into cell culture supernatant showed high levels of oligomannose glycans (73%) and a smaller population of complex glycans (27%). Glycosylation of pelleted pseudoviral $gp120_{JRCSF}$ showed almost no complex glycan processing (<2%). This membrane-associated gp120 shows extremely limited processing by Golgi resident GnT I, as evidenced by the large abundance of the $Man_5GlcNAc_2$ intermediate. The by-pass of GnT I processing appears to correlate directly with high levels of envelope expression in pseudoviral systems, as reduction in envelope level leads to a population of processed complex-type glycans (15%) similar to those observed for shed gp120, indicating that multiple glycoforms are secreted from commonly used pseudoviral expression systems. To relate the glycosylation observed on pseudoviral particles with that present on natural infectious virus, we analysed $gp120_{JRCSF}$ obtained by direct infection of peripheral blood mononuclear cells. The native envelope is composed predominantly of $Man_{5-9}GlcNAc_2$ glycans (79%), with an additional population of core fucosylated, branched sialylated complex-type glycans (21%). Representative viruses from clade A, $HIV_{92RW009}$, and clade C, $HIV_{93IN905}$, exhibited similar glycosylation profiles, indicating the composition of the glycan shield is broadly conserved against different sequences. We also note that the glycosylation of the native envelope spike differs significantly to that of monomeric gp120 typically used in vaccine formulations.

064: Functional Fluorinated MUC1-Glycopetide Vaccines

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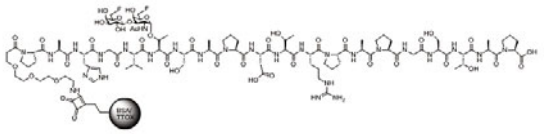
The aberrant glycosylation profiles of mucin glycoproteins on epithelial tumor cells represent attractive target structures for the development of cancer diagnostics and immunotherapy.[1,2] Mucin-type glycopeptides have been successfully investigated as molecularly defined vaccine

prototypes for triggering humoral immunity in animal models.[3] A severe drawback in the development of efficient carbohydrate-based vaccines is the low metabolic stabilities of their glycosidic bonds, which are easily cleaved by endogenous glycosidases. To enhance the bioavailability of the antigenic glycan structure, a hydrolysis-resistant tumor-associated TF antigen analog with fluorine substituents at positions C6 and C6' was synthesized and incorporated into the tandem repeat sequence of the mucin MUC1.[4] The resulting pseudo-glycopeptide was further conjugated to tetanus toxoid (TTox) as a carrier protein and subjected to immunization studies.[5] The difluoro-TF-antigen-MUC1-TTox vaccine induced very strong immune responses in mice with antibodies strongly binding to breast cancer MCF-7 cells.



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065: Pieces of the fungal galactomannan biosynthesis jigsaw (Keynote)

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The filamentous fungus *Aspergillus fumigatus* is the principal cause of invasive aspergillosis, a severe and often fatal disease affecting immunocompromised patients. To tackle this disease, physicians dispose of a limited antifungal arsenal consisting of amphotericin B and various azoles that target the principal membrane sterol, and echinocandins that interfere with the biosynthesis of β-glucan, a major cell wall polysaccharide. Increasing drug resistance contributes to the high rate of therapeutic failure and underlines an urgent need for a new generation of antifungal agents. Besides β-glucan, *Aspergillus fumigatus* cell wall backbone contains the β1,4 N-acetylglucosamine polymer chitin, and galactomannan, a polymer of mannose and galactofuranose. Like echinocandins, inhibitors of chitin synthesis display antifungal activities. In contrast, the biosynthesis of galactomannan and its importance for fungal growth or pathogenicity still remain to be defined. According to our hypothesis, galactomannan is synthesized in the organelles of the secretory pathway on a GPI-anchor and then transferred to the cell wall by transglycosylation or secreted into the extracellular environment. Emerging evidence for this biosynthetic model and the importance of galactomannan for fungal growth will be presented.

Biosynthesis and Metabolism of Glycoconjugates I (Thursday)

066: Glycosylation of Thrombosponsin Type 1 Repeats (Keynote)

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Thrombospondin type 1 repeats (TSRs) are small cysteine rich domains found in a number of cell surface and secreted proteins. TSRs can be O-fucosylated on the hydroxyl of a serine or threonine in the consensus sequence, C-x-x-(S/T)-C-x-x-G. Over 50 proteins in the mouse or human databases contain proteins with at least one TSR containing this sequence, although only a handful have been confirmed to be modified. We have identified the fucosyltransferase responsible for adding fucose to these sites, Protein O-fucosyltransferase 2 (Pofut2), a homolog of the enzyme that adds O-fucose to EGF repeats (Pofut1). Elimination of Pofut2 in a mouse results in embryonic lethality during gastrulation. The mutant embryos show a variety of defects including an enhanced epithelial-to-mesenchymal transition (EMT). The O-fucose on TSRs can be elongated by a β3-glucosyltransferase to generate the disaccharide, Glc-β1,3-Fuc. Mutations in the β3-glucosyltransferase cause Peters Plus Syndrome, a genetic disorder in humans characterized by short stature, developmental delay, eye-chamber defects, and cleft-palate/lip. We are interested in determining the molecular basis for the defects seen in the Pofut2 null mice and Peters Plus patients. To this end, we are examining the predicted targets of Pofut2 to see which are actually modified. We have preliminary data that O-fucosylaton of TSRs, a process that appears to occur in the ER, is required for secretion of several of the proteins known to be modified. Since several Pofut2 targets are proteases known to remodel extracellular matrix (ADAMTS proteases), elimination of Pofut2 may cause a global secretion defect of these proteases. We are examining this and other potential mechanisms to determine the biological function of this unusual form of glycosylation.
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067: A New Enzyme, Glucan Pathway and Anti-TB Target

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We have identified the activity of a bacterial enzyme, GlgE, to be a novel (1-4)-α-D-glucan:phosphate α-D-maltosyltransferase that catalyses the transfer of maltose from maltose 1-phosphate to maltooligosaccharides in a reverse phosphorolysis reaction. It is also capable of a transglucosylation reaction that disproportionates maltooligosaccharides. This enzyme is of the EC 2.4.1 type and despite catalysing glycosyl transfer reactions, it is a GH13_3 subfamily member - the first of which to be biochemically characterised. We have established that GlgE is an alpha retaining enzyme, as expected for a GH13 enzyme. However, GlgE is unusual because it is an anabolic enzyme capable of polymer formation from disaccharide units without the need for a nucleotide diphospho donor. Using an acceptor analogue, we have established that acceptors are extended at their non-reducing ends. The structure of GlgE reveals a core typical of a GH13 family member together with novel features. Ligand-bound structures together with inhibition studies have identified the donor and acceptor binding sites. GlgE is the defining enzyme of a new four-step metabolic pathway in bacteria responsible for α-1,6-branched α-glucan biosynthesis from trehalose. This pathway seems to be responsible for cytosolic carbon management during the development of the differentiating bacterium *Streptomyces coelicolor*. By contrast, it may be responsible for the biosynthesis of the alpha-glucan component of the capsule that not only coats the cell walls of mycobacteria but is also implicated in immune system evasion. We have therefore dissected the essentiality of the genes associated with this pathway in mycobacteria in vitro and in a mouse model using both classical and chemical reverse genetics. This has allowed us to identify GlgE as a potential new drug target in tuberculosis that exhibits a novel mode of action. The discovery of a new target is timely given the lack of new drugs in recent decades to tackle a major gobal infectious disease.

068: Emerging roles for N-glycan de-mannosylation in plants

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In eukaryotes, class I α-mannosidases are involved in N-glycan processing and in endoplasmic reticulum-associated degradation (ERAD) of glycoproteins. To understand the biological function of these enzymes in plants we have characterised class I α-mannosidases (MNS) from *Arabidopsis thaliana*. MNS3 displays a Golgi-like subcellular localization and efficiently cleaves off a single α1,2-mannose residue from the middle branch of $Man_9GlcNAc_2$ indicating that it has a processing function similar to mammalian ER-α-mannosidase I. MNS1 and MNS2 are Golgi-α-mannosidase I proteins and act downstream of MNS3. MNS1 to MNS3 deficiency resulted in the formation of aberrant N-glycans demonstrating that these three MNS proteins play a key role in N-glycan processing. The *mns1 mns2 mns3* triple mutant revealed the almost exclusive presence of $Man_9GlcNAc_2$ and displayed short, radially swollen roots with drastically altered cell walls. One possible explanation for this phenotype is a block in ERAD of glycoproteins. To test this hypothesis we analysed the involvement of MNS proteins in ERAD. The Arabidopsis *bri1-5* mutant displays a severe growth defect that is caused by ERAD of a mutant form of the brassinosteroid receptor BRI1. Pharmacological inhibition of class I α-mannosidases and a loss-of-function mutant (*mrh1*) of an ER-resident mannose-6-phosphate receptor homology domain protein suppress the dwarf phenotype of *bri1-5* by blocking BRI1 degradation. However, mns1 to mns3 mutants fail to suppress the severe growth defect of *bri1-5*. In addition, the identified ERAD mutant *mrh1* displays no root growth phenotype. Together, our data suggest that in contrast to mammals and yeast the Arabidopsis class I α-mannosidases MNS1 to MNS3 are not involved in glycoprotein ERAD and the root growth phenotype of the *mns1 mns2 mns3* mutant is caused by a defect in N-glycan processing.

069: Structure-function studies of galactolipid synthases

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Mono- and digalactosyldiacylglycerol (MGDG and DGDG) are essential components of plastids membranes and constitute the most abundant lipids on earth. Discovery of an apicoplast - a relic of an algal plastid inherited from a secondary endosymbiotic event - in *Plasmodium falciparum* and *Toxoplasma gondii* raised the question of the presence of galactolipids in some pathogenic parasites belonging to the Apicomplexa phylum and opened opportunities to develop new therapeutic drugs. In vitro synthesis of galactolipids could be measured and lipids with a digalactosyl polar head could be detected by immunostaining with an anti-DGDG antibody (1, 2). Despite the biochemical demonstration of galactolipid synthase activities, no gene could be identified so far in the parasite genomes. In contrast the genes coding for the galactolipid synthases in plants have been well

characterized. We conducted structure-function studies on the plant enzymes, particularly on the MGDG synthases (MGD1, MGD2 and MGD3) from *Arabidopsis thaliana*. Much effort has been put into the development of an efficient heterologous expression system to overproduce these enzymes and to set up a purification scheme. In the meantime, 3D models of MGD synthases have been built by homology modeling making possible further analysis. The 3D model of MGD1 has been substantiated by a series of site-directed mutagenesis experiments that gave indication on donor and acceptor binding sites (3, 4). The model also permitted to propose a membrane association protein site and to explore potential binding sites for phosphatidic acid (PA) and phosphatidic glycerol (PG), two anionic lipids that activate MGD1, possibly coupling phospholipids and galactolipid syntheses.

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Biosynthesis and Metabolism of Glycoconjugates II (Thursday)

070: Prokaryotic flagellar glycoconjugates - structural diversity and novel biosynthetic pathways (Keynote)

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Extensive structural analysis of flagellins from a number of pathogenic bacterial species has revealed the presence of a variety of O-linked glycans of diverse and novel structure. In some bacterial species the presence of these glycans are essential for flagella formation and consequent motility. As motility is a critical virulence factor, the glycan biosynthetic pathway offers potential as a novel therapeutic target. The flagellins of the gastrointestinal pathogens *Campylobacter jejuni* and *Helicobacter pylori* are glycosylated with novel sialic acid-like 5,7-diacetamido-3,5,7,9-tetradeoxy-nonulosonate monosaccharides and we have recently determined the biosynthetic pathways for two of these novel sugars, namely pseudaminic acid and legionaminic acid. The synthesis of these two novel bacterial nonulosonate sugars hs been completed in vitro enzymatically and metabolomics based approaches have been used to identify novel biosynthetic genes and to determine the structure of additional derivatives found on the flagellins of *Campylobacter jejuni* isolates. The potential of pathway enzymes as therapeutic targets for antimicrobial drug discovery is currently being explored. Biological roles for these novel sugars in host-pathogen interactions and their utility in glycobiology applications are under investigation. Archaeal species can also produce glycosylated flagellins. In contrast to the bacterial system, archaeal flagellar glycans are attached via N-linkage and the assembly pathway of the flagellar glycoconjugate is distinct.

071: The glycosylation in Nucleo-Cytoplasmic Large DNA Viruses (NCLDV).

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Nucleo-Cytoplasmic Large DNA Viruses (NCLDV) comprise a heterogeneous group of viruses, characterized by very large genomes (0.3 to 1.2 Mbp) infecting several types of eukaryotic cells. In particular Chloroviruses and Mimivirus encode at least part, if not all, of the machinery required to glycosylate their structural proteins. In contrast, most viruses use the host ER/Golgi system for glycoprotein production and their glycan structures are completely dependent on their host cells. Several Chlorovirus-encoded proteins involved in glycoconjugate formation have been identified, including enzymes for nucleotide-sugar production (GDP-L-fucose and UDP-L-rhamnose) and hyaluronan and chitin synthesis, as well as glycosyltransferases and polysaccharide-degrading enzymes. In order to characterise viral glycoconjugates, we have determined the monosaccharide composition of six Chloroviruses, which infect green unicellular algae, and the giant Mimivirus, which infects free-living Acanthamoeba species. Results from Chloroviruses indicate that differences in glycan composition can even differ among viruses that infect the same host, further confirming that glycosylation of the capsid proteins is host-independent. Analyses of Mimivirus indicate that the major sugar component is associated with the long fibers that cover the viral particles. These glycan structures probably contribute to the Gram positive stain displayed by these virions. Identification of the constituent monosaccharides represented the first step for the further structural characterization and has allowed us to identify other virally encoded enzymes, which are involved in modified sugar production and which are currently under study in our laboratories. Studies on the enzymes involved in glycoconjugate production encoded by NCLDVs could provide important insights not only about their role in viral life cycles, but also, due to the long evolutionary history of these viruses, about the relationships with bacterial glycans and with the evolution of the eukaryotic glycosylation machinery.

072: Metabolism of sialic acid (Sia) in protochordates: Structural elements for the Sia recognition and intracellular localization of the CMP-Sia synthetases (CSSs)

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Sialic acid (Sia) is widely distributed, ranging from bacteria to vertebrates. CMP-Sia is a substrate of sialyltransferases that is synthesized by the CMP-Sia synthetase (CSS): Sia + CTP → CMP-Sia + PPi. CSS has two interesting features that have remained unexplained. First, CSS shows preferential recognition of particular Sia species (Neu5Ac, Neu5Gc, and Kdn) depending on animal species. Second, the vertebrate CSSs are localized in nucleus. In this study, we focused on underlying mechanisms for these features: *(1) Sia species-recognition element (SRE)*: Our previous studies on chimeric and site-directed mutants of mouse and rainbow trout CSSs identified the SRE with 16-17 amino acids in the catalytic domain. To ask how conserved the SREs are in various animals, we cloned CSS genes from fish, protochordates, and echinoderm, and measured their enzyme activity using recombinant wild-type and mutated CSSs. The results suggest that the interaction between SRE and the C-5 substituent of Sia is critical for Neu5Ac recognition. Interestingly, for Kdn recognition, the upstream region of SRE may also be involved in the recognition of the 5-OH of Kdn; *(2) Nuclear localization signal (NLS)*: In our previous study, the functional NLS was identified in the middle and N-terminal regions of the catalytic domain of mouse and rainbow trout CSSs, respectively. In this study, the deduced amino acid sequences of cloned CSSs from various animals suggest that nuclear localization of CSS seems to start at echinoderm. This study was supported in part by JST CREST and JST SICP.

073: Sialic acid biosynthesis in *Aliivibrio salmonicida*

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Sialic acids are nine carbon sugars found associated with both bacterial and eukaryal cells. They are vastly important for cell-cell communication, pathogen interaction and immune recognition. Sugar-modifying enzymes can be utilized in many different areas in molecular biology and medicine. The combination of chemical- and enzymatic synthesis will bring forward new saccharides which can have useful properties. The genome of the psychrophilic and fish pathogenic bacterium *Aliivibrio salmonicida* LFI1238 reveals that it possesses the ability to synthesize two sialic acids, neuraminic acid and legionaminic acid. We have expressed and purified several of the proteins belonging to the pathways leading to these sugars and two protein structures have been solved. The aims of this study are to characterize and determine 3D-structures of more of the enzymes synthesizing sialic acids in *A. salmonicida*, and to figure out if they are important for bacterial survival in the host. Knock out of genes from these two pathways might give answers about their functions and possible roles in virulence. Since *A. salmonicida* is a psychrophile it might produce cold-adapted versions of the sialic acid synthesizing enzymes. Cold-adapted enzymes are often known to be more efficient compared to mesophilic counterparts, which renders them highly interesting as targets for commercial exploitation. The commercial potential will be further investigated through the characterization of the enzymes. Assays and methods for purifying and analyzing the products of the enzymatic reactions are being developed.

Biosynthesis and Metabolism of Glycoconjugates III (Thursday)

074: A novel metabolic pathway for N-linked glycans (Keynote)

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There is growing evidence that N-linked glycan play pivotal roles in protein folding and intra- or intercellular trafficking of N-glycosylated proteins. It has been known that during the N-glycosylation of proteins, significant amounts of free oligosaccharides (fOSs) are generated in the lumen of the endoplasmic reticulum (ER) by an unclarified mechanism. fOSs are also formed in the cytosol by enzymatic deglycosylation of misfolded glycoproteins destined to be destroyed by a cellular system called ER-associated degradation. Although the precise fate of intracellular fOSs remains obscure, recent biochemical studies have revealed that a yet-unexplored cellular machinery is involved in formation/degradation of fOSs. It has been shown that the cytoplasmic peptide:N-glycanase (PNGase), endo-β-N-acetylglucosaminidase (ENGase), and mannosidase (Man2C1) constitutes cytosolic enzymes involved in formation/processing of the cytoplasmic fOSs in mammalian cells (1-3). There are several players involved in this basic biological process, however, remained to be identified. Moreover, recent data suggested that molecular mechanism of fOSs metabolism appears quite distinct between species (4). In this

presentation, I will introduce our new findings on formation, processing and degradation of fOSs in mammalian cells as well as in yeast.

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075: Metabolic glycoengineering through the mammalian GalNAc salvage pathway.

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Glycans are frequently present at the surface of cancer cells in a different form compared to healthy cells. However, these tumor associated carbohydrate antigens do not lead to an effective immune response. GalNAc is the first sugar of mucin type O-glycans, is present on N-glycans, glycolipids and proteoglycans and is a component of several well characterized tumor antigens like GD2 and the Tn-, SiaTn- or T-antigens. The aim of this work was to prepare synthetic GalNAc analogs which can be incorporated into glycans at the surface of cancer cells and into mucins synthesised by tumors in order to analyze their potential as targets for an immune therapy against those modified tumor associated carbohydrate antigens. GalNAc analogs were chemically synthesized and were tested in vitro as substrates of the enzymes constituting the mammalian GalNAc salvage pathway: the human galactokinase (GK2) and the human UDP-pyrophosphorylase (AGX1). The best candidates allow the synthesis of the corresponding UDP-sugars which are further used to transfer those analogs onto peptides and proteins using a bovine GalNAc transferase (ppGalNAc T1). We show that some of the synthetic analogs can be readily integrated into the GalNAc salvage pathway and transferred to Ser or Thr in peptides and proteins. Most of the glycoproteins carrying GalNAc analogs are substrates for the core 1 Gal transferase as well as for ST6GalNAc1. Moreover, mammalian cells were cultivated in the presence of these analogs and their incorporation into glycoconjugates at the cell surface was analyzed by FACS using appropriate fluorescent lectins and antibodies.

076: Genetic analysis of de novo and salvage pathways for the nucleotide sugar UDP-glucuronic acid

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Cell wall polymers are synthesized using nucleotide sugars as glycosyl donors. The main precursor for leaves from Arabidopsis is UDP-glucuronic acid, providing approximately 50% of the cell wall biomass, as it is the common precursor for apiose, arabinose, galacturonic acid and xylose in polymers. We are studying two de novo pathways for the synthesis of UDP-GlcA, involving UDP-glucose dehydrogenase (UGD) and myo-inositol oxygenase (MIOX) as key enzymes. Both enzymes are encoded by a small gene family with four members each. The analysis of knockout mutants indicates a dominant role of UGDs for precursor biosynthesis of cell wall polymers. A reduction of the UDP-GlcA supply results in modified cell walls, causing developmental defects and growth problems. A reduction in MIOX activity is compensated by the UGD pathway and therefore causes no changes in the cell wall of miox knockdown mutants. However miox mutants show metabolic phenotypes and most interestingly they are more resistant to nematode infections. We have recently also focused on a salvage pathway for UDP-GlcA, which is interconnected with the MIOX pathway. A knockout in the terminal enzyme UDP-sugar pyrophosphorylase is pollen lethal and therefore homozygous knockout plants cannot be obtained. Using various genetic complementation approaches we circumvented this problem and are now able to study mutants in the salvage pathway with only minimal residual activity. The analysis of these mutants surprisingly reveals a much more important role of nucleotide sugar recycling as previously thought.

077: C-terminus Glycans with Critical Role in the Maturation of a Secretory Glycoprotein

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The N-glycans of membrane glycoproteins are mainly exposed to the extracellular space. Human tyrosinase is a transmembrane glycoprotein with six or seven bulky N-glycans exposed towards the lumen of subcellular organelles. The central active site region of human tyrosinase is modeled here within less than 2.5Å accuracy starting from *Streptomyces castaneoglobisporus* tyrosinase. The model accounts for the last five C-terminus glycosylation sites of which four are occupied and indicates that these cluster in two pairs - one in close vicinity to the active site and the other on the opposite side. We have analyzed and compared the roles of all tyrosinase N-glycans during tyrosinase processing with a special focus on the proximal to the active site N-glycans, s6:N337 and s7:N371, versus s3:N161 and s4:N230 which decorate the opposite side of the domain. To this end, we have constructed mutants of human tyrosinase in which its seven N-glycosylation sites were deleted. Ablation of the s6:N337 and s7:N371 sites arrests the post-translational productive folding process resulting in terminally misfolded mutants subjected to degradation through the mannosidase driven ERAD pathway. In contrast, single mutants of the other five N-glycans located either opposite to the active site or into the N-terminus Cys1 extension of tyrosinase are temperature-sensitive mutants and recover enzymatic activity at the permissive temperature of 310°C. Sites s3 and s4

display selective calreticulin binding properties. The C-terminus sites s7 and s6 are critical for the endoplasmic reticulum retention and intracellular disposal. Results herein suggest that individual N-glycan location is critical for the stability, regional folding control and secretion of human tyrosinase and explains some tyrosinase gene missense mutations associated with oculocutaneous albinism type I.

Structural and Chemical Glycobiology and Glycomics I (Thursday)

078: The role of oligosaccharides in protein-protein interactions

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We have analyzed interactions of viral proteins with receptor proteins on human cells to elucidate the roles of carbohydrate components of glycoproteins during the binding process of the virus to the human host cell. Normally, virus proteins that are located on the surface of the virus are glycosylated with O- and N-type in glycan chains. A large glycoprotein gp120 is found on the surface of HIV. It has two essential interactions with the receptor proteins on human cells, CD4 and CCR5. Both the gp120 and the receptor proteins are glycosylated. In the case of GP 120 about 50% of the molecular weight is formed by 26 oligosaccharide chains. We analyzed both interactions by NMR and surface plasmon resonance. Modeling yielded the concept for the understanding of these protein protein interactions. In the case of the interaction of CD4 with GP120 we also synthesized mimetics to block this contact. In both cases the dominant binding motif is the peptide peptide interaction. However, glycosylation plays in each case the role in terms of improving binding affinity by a factor of roughly 500. It also increases the on rate of the cycle at tides in their interaction with the receptor proteins. The role of the carbohydrates in forming this interaction and the increase in association rate will be discussed.

079: NanoLC-MS/MS methods for O-glycome analysis

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Analysis of protein glycosylation is crucial in establishing the glycans' functions in health and disease. Generally, two strategies can be employed: 1.) A glycoprotein can be digested and the resulting glycopeptides further analyzed for glycan structural elucidation and identification of the glycosylation site. 2.) Releasing methods for oligosaccharides can be applied prior to glycan analysis. Our aim is to develop suitable strategies for O-glycan detection which can be combined with N-glycosylation analysis. Two different approaches are described: (1) A protocol for glycopeptide dissection (in solution or in gel) was developed in which fetuin was subjected to unspecific proteolysis by Proteinase K or Pronase. The resulting (glyco)-peptide samples were resolved by nanoHPLC-ESI-ion trap (IT)-MS. Three different separation principles, hydrophilic interaction liquid chromatography (HILIC), C18-reverse phase (RP), and graphitized carbon HPLC, were applied, and the results compared. MS/MS and MS^3 spectra served for oligosaccharide and peptide analysis. All results were in accordance with the known site-specific N- and O-glycosylation pattern of fetuin. Nanoscale HILIC-online MS yielded the best results for the combined analysis of N- and O-glycopeptides. (2) We developed a combined O-glycan release and derivatization protocol followed by mass spectrometric analysis. O-glycans from glycoproteins were released using dimethylamine (DMA) in the presence of 1-phenyl-3-methyl-5-pyrazolon (PMP), resulting in a 2:1 stoichiometry of PMP per glycan. Samples were worked up by vacuum centrifugation and solid phase extraction, followed by nano-LC-ESI-IT-MS/MS analysis using a conventional RP nanocolumn. In conclusion, we here present two methods that allow (1) the analysis of glycopeptides generated by broad-specificity proteinases for O- and N-glycan analysis, and (2) a combined approach of O-glycan release and labeling for a fast, comprehensive, and sensitive analysis of O-linked oligosaccharides.

080: The major glycosylphosphatidylinositol- (GPI)-linked surface antigen of the protozoan parasite *Entamoeba histolytica*

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A major part of the surface coat of the intestinal protozoan parasite *Entamoeba histolytica* consists of glycosylphosphatidylinositol- (GPI)- linked molecules. Based on a measurement of inositol, we estimated around 100 million of such molecules per cell. These molecules have been called proteophosphoglycans (PPGs), lipophosphoglycans (LPGs) or lipopeptidophosphoglycans (LPPGs). Moody-Haupt et al. showed in 2000, that the molecules may be GPI-linked glycoproteins with a number of serine-linked oligoglucose side chains. The monoclonal antibody EH5, raised in our laboratory, bound to these antigens and gave significant passive protection in a SCID mouse liver abscess model. In gel electrophoreses,

the antigens migrated as a broad smear. To investigate the EH5 epitope, we identified a number of peptide mimotopes from several phage display libraries, which revealed a single consensus motif GTHPXL. The *E. histolytica* genome contains one gene coding for a 52 kDa polypeptide comprising exactly this consensus sequence. So far, our efforts to cleave and analyse the core protein from the isolated antigen preparation have been unsuccessful. The recombinant protein, generated at low levels in *E. coli*, gave strong western blot signals with the EH5 antibody. The short peptide sequence, genetically fused to a foreign protein, was recognised by the EH5 antibody, confirming that the epitope was a sequential protein epitope. Interestingly, the *E. histolytica* gene had a weak but significant similarity to the CD68/macrosialin antigen, a glycoprotein which is found on the surface and in vesicles of mammalian phagocytic immune cells such as macrophages and neutrophilic granulocytes. The EH5 antibody inhibited the phagocytosis of erythrocytes and bacteria by *E. histolytica*. Supported by grant BIO4-CT98-0121 from the European Commission and grants P15960 and P22037 from the Austrian Science Fund.

081: The role of the membrane protein Wzi in capsule assembly (Keynote)

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Pathogenic bacteria frequently cloak themselves in a carbohydrate rich capsule. The carbohydrate building blocks are assembled in the periplasm. In *E. coli* K30 the carbohydrates are polymerised into a tetrasaccharide repeat. The repeat is translocated across the cytoplasmic membrane where it is polymerised into a long chain. The long chain is translocated across the outer membrane by the protein Wza. The structure of Wza was determined some years ago. The protein Wzi has been identified by Whitfield and co-workers are important but not essential for capsule formation. Genetic knockout data suggest it plays a role in anchoring capsule to the cell, disruption of the capsule is a potential therapeutic target. This talk will focus on recent biochemical and structural analysis of Wzi, these studies suggest the molecular basis for its function.

Structural and Chemical Glycobiology and Glycomics II (Thursday)

082: A novel technique for constructing oligosaccharide microarrays

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We believe the best tool for detecting carbohydrate related diseases is oligosaccharide microarray (glycochip). The construction of oligosaccharide microarray is dramatically developing resent years. Unsurprisingly, more carbohydrate structures detect more carbohydrate related diseases. In order to construct unlimited carbohydrate structures on a chip, we developed a novel technique for constructing oligosaccharide microarrays. As same manner as to construct DNA chip, we applied photolithography technique to construct oligosaccharide microarray as on-chip synthesis. Selective synthesis of carbohydrates on a chip succeeded with the combination usage of uni-chemo hydroxyl protection procedure[1] and a photo-labile protecting group. The reaction of on-chip synthesis was successfully monitored with MALDI-TOF MS after cleavage from the chip.[2] The chip was successfully recognized by a lectin.

1. Komba, S.; Terauchi, T.; Machida, S. J. Appl. Glycosci. 2011, 58, 1-12. 2. Terauchi, T.; Machida, S.; Komba, S. Tetrahedron Lett. 2010, 51, 1497-1499. This work was supported by the Precursory Research for Embryonic Science and Technology (PRESTO) program.

083: Characteristics of the CFG printed glycan microarray v5.0: final glue-grant funded

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The CFG glycan microarray screening has been a prevailing technique in determining GBP binding specificity and glycan ligand identifications that has been available, free-of-charge, to the community(1). The recently released CFG Glycan Microarray (v5.0) comprises 611 glycans ranging from 1 to 37 residues. Version 5.0 boasts an unprecedented diversity of compounds, encompassing linear and multi-branched core structures of synthetic and natural N-linked, O-GalNAc, O-mannosyl and glycolipid glycan derivatives. Core structures are elaborated with various terminal motifs of sialylated, fucosylated and sulfated glycans on type 1 and type 2 lacNAcs, gangliosides, globoseries and ABO blood group antigens, as well as the newly-added extended polyLacNAc structures on natural glycans and HNK-1 antigens.

Over 70% of compounds have been chemoenzymatically synthesized in-house, and the remaining were contributed by N. Bovin's and J. Paulson's laboratories as well as several other investigators. All of the compounds are immobilized on an NHS-activated hydrogel slide via amide bond formation with functional amines generated at the glycan's reducing-end. The glycans are printed at 100 μM concentration in replicates of six on the glass slide. Every batch of the printed array undergoes two levels of screening for quality control (QC): a high concentration 17-lectin analysis "Standard QC" and, a low concentration

4-lectin method "Release QC".

1) www.functionalglycomics.org
* Funded by NIGMS grant GM62116

084: A Novel Class of Glycosyltransferase Inhibitors

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Glycosyltransferases (GTs), Nature's "glycosylation reagents", are a large family of carbohydrate-active enzymes which catalyse the transfer of a sugar from a specific glycosyl donor to a suitable acceptor [1]. GTs are critically involved in many fundamental biological processes, such as the biosynthesis of cellular adhesion molecules and bacterial cell wall biosynthesis. Inhibitors of GTs are therefore sought after as chemical tools for the investigation of these processes and as lead compounds for drug discovery [2]. We have recently discovered a new type of GT inhibitor with a novel, allosteric mode of action and potent activity against a panel of different galactosyltransferases (GalTs) [3]. The prototype inhibitor, a base-modified analogue of the GalT donor substrate UDP-galactose (UDP-Gal), blocks a conformational change in the active site of a representative GalT, which is critical for catalytic activity. In this presentation, we report the latest results from our ongoing studies into the scope and structural basis for this new mode of GT inhibition. We present new analogues of the prototype inhibitor, including a hybrid derivative which incorporates an additional inhibitor motif. We also discuss different strategies for the development of membrane-permeant GT inhibitors in this class, including preliminary results from cellular studies. The delivery of the new inhibitors across biological membranes is a particularly important challenge, with a view towards their application as chemical tools for glycobiology. Financial support of this research by the MRC (G0901746) and the BBSRC (BB/H024433/2) is gratefully acknowledged.

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085: Glycoarrays on Gold Surfaces (Keynote)

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Self-assembled monolayers (SAMs) on gold have become widely used as an attractive platform for studying chemical and biochemical reactions, for studying biomolecular interactions and for the development of nanoscale devices. We have used the platform to study the solid-supported synthesis of carbohydrates and glycopeptides using both chemical and enzymatic methods. An attractive feature of the technology is the opportunity for miniaturisation and in situ analysis using mass spectrometry, SPR and fluorescence spectroscopy. Applications for the synthesis of complex oligosaccharides and glycopeptides to generate glycoarrays and their application in biology and medicine will be discussed.

References: Laurent et al, ChemBioChem (2008) 9, 883-887; Zhi et al, ChemBioChem (2008) 9 (10) 1568 – 1575; Laurent et al, Trends in Biotechnology (2008) 26 328-337; Laurent et al, Chem. Commun. (2008) 4371-4384; Laurent et al, ChemBioChem (2008) 9, 2592-2596; Deere et al, Langmuir (2008), 24(20), 11762-1176; Haddoub et al, Org. Biomol. Chem. (2009) 7, 665–670; Voglmeir et al, OMICS J. (2010) 14 (4), 437-444; Voglmeir et al, Biochemical J. (2011) in press; Sardzik et al, Chem. Commun. (2011) in press.

Structural and Chemical Glycobiology and Glycomics III (Thursday)

086: In-depth Characterization of Glycoproteins by ZIC-HILIC Enrichment, Mass Spectrometry and Glyco-Bioinformatics

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Glycosylation is the most abundant protein posttranslational modification and is involved in many relevant biological processes and crucial to the understanding of many diseases. In depth analysis of glycosylation sites is difficult, however, as glycopeptides exhibit a significant micro heterogeneity at glycosylation sites. In addition, ion suppression effects require selective methods for glycopeptide enrichment. Mass spectrometric analysis of glycopeptides is challenging because both the peptide as well as the glycan moiety have to be elucidated for a full structural understanding. We used fetuin, asialofetuin and alpha-1-acid glycoprotein as sources of sialylated and non-sialylated glycosylated structures. In addition, monoclonal antibodies were analyzed as a dedicated example for pharmaceutical QC. Proteins were digested with trypsin and glycopeptides were enriched using a dedicated ZIC-HILIC glycocapture beads in combination with an optimized buffer system (EMD Chemicals Inc.). The glycopeptides were analyzed using ESI ion trap MS for glycoprofiling and MALDI-TOF/TOF-MS for in depth characterization of the glycopeptides. For database searches, an integrated software approach was used: protein searches of

the glycopeptide MS/MS spectra were performed for obtaining the amino acid sequence of the glycopeptide, and searches in glycan databases based on the same glycopeptide MS/MS spectra were carried out to complete the characterization of N-linked glycopeptides. Compared to MS analysis of native glycoprotein digests, the enriched samples allowed the detection of more glycopeptides and permitted the acquisition of higher quality MS/MS spectra. For MALDI-TOF/TOF-MS analysis, linear positive ion mode detection of precursor ions proved to be highly suitable for the analysis of even multi-sialylated glycopeptides. The employed integrated software approach allowed glycan identification to function similarly to that for peptide identification. The important step of interactive result validation was facilitated by a suite of dedicated data and result viewers. Overall the software support in glycoprotein analysis greatly facilitated the analysis and reduced the time required to assign glycoprotein structure.

087: Systems Glycobiology: from Genome to Glycome An Integrated Strategy for Identifying and Screening Potential Clinical Markers

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As more therapeutic options become available there is an increasing need for clinical markers that will provide more sensitive and specific early detection of disease. At the same time, improved technologies for monitoring disease progression and response to therapy are required. In many cases, single assays of existing biomarkers are neither sensitive nor specific enough for use as sole screening methods and in general a combination of markers is required. Many systemic diseases, particularly cancer, have been linked with many systems, from genomics to glycomics. Therefore we have developed an automated 96-well plate based strategy for identifying, quantifying and screening potential glycans released from proteins in body fluids as clinical markers. We have constructed a data base of the serum glycome of healthy controls to compare with that of clinical controls and of patients with various diseases including schizophrenia, rheumatoid arthritis, breast, ovarian, lung, stomach, prostate and pancreatic cancers and compared the specificity and sensitivity of the glycan markers with the current markers used in the clinics. Automated data analysis is subsequently fine tuned for each disease opening the way for undertaking large scale clinical trials that may prove useful for diagnosing disease and monitoring progression and therapy. Importantly, the technology has enabled links to be made from the serum glycome to individual glycoproteins, glycoprocessing pathways, signaling transduction pathways and to the genome itself, demonstrating the possibility of probing a whole system for disease associated changes and providing a deeper insight into pathogenesis.

088: High-Throughput Glycosylation Pattern Analysis of Glycoproteins Utilizing a Multiplexing Capillary-DNA-Sequencer

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Glycomics is a rapidly emerging field that can be viewed as a complement to other „omics“ approaches including proteomics and genomics. Hence, there is a dramatic dynamic increase in the demand for sophisticated databases and analytical tools in glycobiology respectively glycobiotechnology. In order to enhance and improve the comparatively small existing glycoanalytical toolbox, fully automated high-throughput (HTP) and high-resolution analysis methods including automated data evaluation are required. Especially one glycoanalysis approach, based on multiplexed capillary gel electrophoresis with laser induced fluorescence detection (CGE-LIF) utilizing a DNA-sequencer, shows high potential for HTP glycoprofiling of glycoconjugates [1,2,3].

The aim of the project presented was to further investigate and to improve this innovative approach for different fields of application with respect to sample preparation, separation and data analysis. First, sample preparation method and workflow were further optimized with respect to performance and feasibility regarding HTP. Second, with up to 96 capillaries in parallel, the fully automated separation with an impressive sensitivity is shown to result in massive reduction of the effective separation time per sample. Third, data analysis was automated developing a novel modular software-tool for data-processing and -analysis, interfacing a corresponding oligosaccharide-database. Using this software-tool, the generated “normalized” electropherograms of glycomoieties (“fingerprints”) can be evaluated on two stages: “simple” qualitative and quantitative fingerprint comparison and structural elucidation of each single glycocomponent. The smart applicability of this technique is demonstrated for different types of glycosamples such as the “glycome” of (monoclonal) antibodies, human milk and of human blood serum. This novel modular glycoanalysis system and method allows fully automated, highly sensitive instrument-, lab- and operator-independent high-throughput HTP-glycoanalysis, even when operated by non-experts. This is in contrast to the currently prevailing methods, where multiplexing with respect to high-throughput is highly cost and lab-space intensive and ties up a lot of manpower and experts hands-on-time.

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089: Rhamnogalacturonan II exhibits a diversity based on monosaccharide exchanges, undescribed methylation and methylesterifications

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Rhamnogalacturonan II (RGII) is the most complex polysaccharide in plants. The actual idea about RGII is that it possesses a much conserved monolithic structure. We developed a fast analytical method allowing the structural characterisation by LC-ESI-MSMS of all fore side chains released by TFA. RGII structures are much more diverse than originally depicted. First, the α1,4 linked L-fucose of the chain A is partly replaced by an hexose. The level of substitution attains up to 50 % in mung bean sprouts. Results obtained with *mur1* mutant and GME RNAi knock down plants show that this hexose is L-galactose. NMR analysis coupled with mass spectrometry data of native, base treated or reduced released chain A revealed the presence of methylesterification of its glucuronic acid. This phenomenon concerns all species tested, from moss to angiosperms. Finally one galacturonic acid can be methylated either once or twice. In some species like *N. benthamiana*, chain A with xylose instead of methylxylose is also present. Variability of the chain B is essentially a matter of length and highly truncated chain B are for instance observed in siliques of *A. thaliana*. The presence of a second terminal rhamnose residue on the chain B of this plant, foreseen as being absent, is found, but at a low level. The variability of RGII was confirmed by analysing, accurately for the first time to our knowledge, full monomers of RGII obtained by direct enzymatic digestion of crude cell wall, with the same method. The rapidity of our method makes it suitable for mutant screening.

090: High-throughput glycosylation profiling for clinical glycomics of autoimmune and alloimmune diseases (Keynote)

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Several innovative methods for structural glycomics will be presented, including enrichment and purification of glycans and glycopeptides by cotton HILIC microtips followed by MALDI-TOF-MS analysis [1], protein glycosylation analysis employing broad-specificity proteinases and mass spectrometric characterization of glycopeptides [2], high-throughput (HTP) glycosylation profiling using multiplexed CGE-LIF on a DNA analyzer [3], mass spectrometric IgG glycosylation profiling [4], and a shot-gun glycan microarray approach for the characterization of autoantibodies in cancer [5]. In addition, results will be presented emphasizing the role of glycosylation in the (dys-)regulation of humoral immune responses in autoimmune and alloimmune diseases such as rheumatoid arthritis and fetal-maternal alloimmune thrombocytopenia (FNAIT). [1] Selman et al (2011) Anal Chem, in press [2] Zauner et al (2010) J Sep Science, 33:903 [3] Ruhaak et al (2010) J Proteome Res, 10: 6655 [4] Selman et al (2010) Anal Chem, 82: 1073 [5] Lonardi et al (2010) Expert Rev Proteomics, 7: 761

Cell Biology I (Friday)

091: LOX-1 functions as a common receptor for Hsp60, Hsp70, and Hsp90 and facilitates cross-presentation of apoptotic cell-associated antigens (Keynote)

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Heat shock proteins (HSPs) were originally identified as stress-responsive proteins and serve as molecular chaperones in different intracellular compartments. Translocation of HSPs to the cell surface and release of HSPs into the extracellular space have been observed during the apoptotic process and in response to a variety of cellular stress. However, little is known about the biological function of extracellular HSPs, especially HSPs on the cell surface. Here, we report that Hsp60, Hsp70 and Hsp90 on the apoptotic cells could facilitate the clearance of apoptotic cells and beads coated by Hsp60, Hsp70, or Hsp90 could be uptaken by bone marrow-derived dendritic cells. Furthermore, we identified that LOX-1 is engaged as a common receptor for Hsp60, Hsp70, and Hsp90. LOX-1 could uptake apoptotic cells by recognizing Hsp60, Hsp70 and Hsp90 on the apoptotic cells and abolishing the binding of LOX-1 with Hsp60, Hsp70 and Hsp90 could inhibit the uptake of apoptotic cells. Moreover, dendritic cells could cross-present OVA antigen from apoptotic E. G7 cells on MHC class I molecules via LOX-1 and inhibition of the uptake of apoptotic cells by LOX-1 decreases cross-presentation of OVA antigen. Taken together, these results demonstrate that LOX-1 could uptake apoptotic cells by recognizing Hsp60, Hsp70 and Hsp90 on the cell surface and facilitate cross-presentation of apoptotic cell-associated antigens into MHC class I presentation pathway.

092: Suppression of α-mannosidase (Man2C1) gene expression by siRNA induces mitochondria-dependent apoptosis in HeLa cells

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Cytosolic α-mannosidase, Man2C1, has been shown to be involved in trimming of free oligosaccharides in the cytosol of mammalian cells. It was also reported that suppression of Man2C1 gene expression resulted in growth delay of esophageal carcinoma cells through inducing mitotic arrest and apoptosis. Therefore, Man2C1 can be regarded as an attractive target for anti-cancer drug. At this moment, however, the detailed mechanism of the induction of apoptosis by Man2C1 suppression in mammalian cancer cells remains unknown. In this study, we established stable transfectants of Man2C1 siRNA using HeLa cells and pancreatic carcinoma PK59 cells, respectively. It was revealed that siRNA treatment of Man2C1 caused apoptosis in HeLa cells, but not in PK59 cells. Consistent with this observation, release of cytochrome C from mitochondria to the cytosol, an indication of mitochondria-dependent apoptotic cell death, was evident in HeLa cells, but not in PK59 cells. We found that Man2C1 was expressed at much higher level in PK59 cells compared to that in HeLa cells, which could at least in part account for the different response to siRNA treatment. Strikingly, rescue experiment of Man2C1 siRNA-induced apoptosis by Man2C1 overexpression revealed that even catalytic-inactive form of Man2C1 could rescue the apoptosis of HeLa cells, implying that the regulatory property of Man2C1 might be independent of its mannosidase activity.

093: Structural remodeling of GPI-anchored proteins is critical for their sorting to ER-exit sites by p24 family proteins

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Glycosylphosphatidylinositol (GPI) anchoring of proteins is a post-translational modification occurring in the endoplasmic reticulum (ER). Following GPI attachment, proteins are transported by coat protein complex II (COPII)-coated vesicles from the ER. Since GPI-anchored proteins (GPI-APs) are lumenally localized, they cannot interact with cytosolic COPII components directly. Receptors that link GPI-APs to COPII are thought to be involved in efficient packaging of GPI-APs into vesicles; however, mechanisms of GPI-AP sorting are not well understood. During transport of GPI-APs, lipid and glycan moieties of GPI-anchor are remodeled. Here, we report that two remodeling reactions of GPI anchors, mediated by PGAP1 and PGAP5, are required for sorting of GPI-APs to ER-exit sites. The p24 family of proteins recognizes the remodeled GPI-APs and sorts them into COPII vesicles. Association of p24 proteins with GPI-APs is pH-dependent, suggesting that they bind in the ER and dissociate in post-ER acidic compartments. Our results indicate that p24 complexes act as cargo receptors for correctly remodeled GPI-APs to be sorted into COPII vesicles.

094: The interactions of the *Pseudomonas aeruginosa* lectin LecA with the glycosphingolipid Gb3 result in membrane invagination and cellular uptake of the bacterium

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Pseudomonas aeruginosa is a major human opportunistic pathogen because it is able to cause severe infections of the respiratory and urinary tract, skin and eye. The internalization of *P. aeruginosa* by host cells appears to play a fundamental role in the pathogenesis of this bacterium. However, the molecular mechanisms underlying P. aeruginosa invasion into non-phagocytic cells, and the microbial and host cell factors involved in this process, remain still largely unknown despite several important contributions over the last decades. Based on novel endocytic concepts that we have established for some bacterial toxins and animal viruses, we hypothesize that specific interactions of the *P. aeruginosa* lectin LecA with the glycosphingolipid Gb3 exposed at the host cell surface lead to formation of tubular plasma membrane invaginations, recruitment of cytosolic proteins and cellular uptake of the bacterium. This extension automatically raises the question whether this endocytic strategy rather represents a general concept of pathogen invasion into host cells. We have reconstituted the glycosphingolipid Gb3 into giant unilamellar vesicules (GUVs) and incubated these vesicles either with the *P. aeruginosa* wild-type strain PAO1, the LecA-deficient strain of PAO1 or with the *P. aeruginosa* lectin LecA. The wild-type strain PAO1 bound nicely to Gb3-containing liposomes and induced membrane invaginations, in contrast to the LecA-deficient PAO1 strain. In addition, LecA bound and formed tubular membrane invaginations on Gb3-containing GUVs as well as energy-depleted or dynasore-treated HeLa cells. These observations let us conclude that it is the tetrameric lectin LecA on the bacterial outer membrane that binds specifically to its receptor, the glyocosphingolipid Gb3, and induces the formation of membrane invaginations as the first step of bacterial entry. Understanding the fundamentals of the complex interplay between microbial pathogens and their glycan receptors at the molecular level could lead to the development of novel therapeutics and diagnostics.

Cell Biology II (Friday)

095: Role of N-linked oligosaccharides in protein quality control and in maintenance of endoplasmic reticulum homeostasis (Keynote)

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The endoplasmic reticulum (ER) is the site of maturation for proteins destined to the extracellular space, the plasma membrane and to the organelles of the endocytic and secretory pathways. Most of them are covalently modified at asparagine side chains with a pre-assembled oligosaccharide composed of 3 glucose, 9 mannose and 2 N-acetyl glucosamine residues. Compelling data synthesized in the mannose timer model of glycoprotein quality control reveal that non-native glycopolypeptides are removed from the ER lumen upon extensive de-mannosylation of their oligosaccharides. In the mannose timer model, the mannosidase-like protein EDEM1 and the mannose 6-phosphate receptor homology (MRH) domain-containing lectin OS-9 play a crucial role by processing and/or recognizing oligosaccharides displayed on misfolded polypeptides thereby preparing them for dislocation across the ER membrane. EDEM1 and OS-9 may also use their mannosidase-like and MRH domains for association with SEL1L, a penta-glycosylated component of the dislocation machinery that assist retro-translocation of misfolded proteins across the ER membrane. Our studies aim at understanding the relevance for ER homeostasis and protein quality control of the formation of these lectin-based SEL1L:EDEM1 and SEL1L:OS-9 complexes.

096: Defining the molecular machinery involved in the retrograde transport of Shiga toxin from endosomes-to- the Golgi

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Retrograde transport pathways are integral to the recycling of many membrane proteins and are also exploited by bacterial protein toxins like Shiga toxin for pathogenesis (1). The B subunit of Shiga toxin binds to specific glycolipids on the host cell, namely globotriaosylceramide (Gb3), and is then internalized into endosomes. From the early endosome, Shiga toxin is transported in a retrograde manner to the TGN, through the Golgi stack and eventually reaching the ER. We have mapped the retrograde transport requirements of Shiga toxin and compared it with other cargos, such as TGN38, that use endosome-to-Golgi transport. Tracking the transport of Shiga toxin demonstrated that the bulk of the toxin is transported from early endosomes to recycling endosomes en route to the TGN whereas another cargo, TGN38, is transported directly from early endosomes to the TGN. The use of Shiga toxin has allowed the identification of the molecular machinery which specifically regulates this retrograde transport pathway. We have mapped the requirements of retromer and sorting nexins for sorting and exit of Shiga toxin from the early endosomes, as well as small G proteins, SNAREs and tethers for delivery of Shiga toxin to the TGN. Overall, our findings have identified a distinct set of components responsible for the regulation of the retrograde transport pathway used by Shiga toxin.

Reference: 1. Lieu, ZZ and Gleeson, PA (2011) Endosome-to-Golgi transport pathways in physiological processes. Histol Histopath 26:395-408

097: Galactan of flax phloem fibers is associated with specific protein

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Flax phloem fibers form a gelatinous secondary cell wall. Its development is associated with metabolism of tissue- and stage-specific polymer - galactan. Galactan, built as the complex rhamnogalacturonan I with side chains of ß-(1,4)-galactose, accumulates in the Golgi vesicles before incorporation into the cell wall. In the fraction of galactan isolated at the active synthesis stage from Golgi vesicles, a protein was detected. Elution of the protein (Mw 70 kD) together with polysaccharide (Mw 2000 kD) indicates their association. Separation of protein and galactan by ionic detergent is evidence for the non-covalent nature of this association. Association of galactan with protein allowed to separate the latter from the set of soluble proteins of the flax fiber-enriched peels and to obtain pure preparations of protein. The protein has been identified as ß-galactosidase. Galactan was established to be the substrate for identified galactosidase *in vitro*. Cellular and subcellular localization of the enzyme in flax stem tissues *in vivo* was demonstrated: it is co-localized with tissue-specific galactan in the phloem fibers, forming the secondary cell wall, namely in the Golgi vesicles and in the inner layers of the cell wall. It was established that after galactan incorporation into the cell wall the modifications of the polysaccharide, which involves shortening of the side chains, occur. Post-synthetic modifications of galactan are linked to the formation of a special supramolecular structure of the gelatinous cell wall, which determines the specific functions of plant fibers. The observed association of polysaccharide and the modifying enzyme raises a number of questions. In particular, what inhibits the enzyme when it is associated with galactans in the Golgi vesicles? What is the signal for polysaccharide hydrolysis and how the degree of hydrolysis is controlled? Perhaps the secret lies in the peculiarities of the

polysaccharide structure and its molecular conformation. These aspects are partially unraveled in our work.

098: Golgi Endomannosidase Mediates a Novel Route for Endoplasmic-Reticulum Associated Degradation

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Quality control in the endoplasmic reticulum is a cellular process during which terminally misfolded proteins in the lumen of the ER are recognised and targeted for ER-associated degradation (ERAD). Quality control is closely linked to N-glycosylation, which has been shown to play a regulatory role in determining the fate of newly synthesised glycoproteins. The classic view on ERAD attributes the major roles in glycoprotein quality control and ERAD targeting solely to ER-localised enzymes and lectins. Multiple reports, however, suggest the involvement of post-ER compartments in glycoprotein degradation. Golgi/ERGIC-resident endo-α-mannosidase is known to provide a pathway for deglucosylation of glycoproteins, alternative to that mediated by ER glucosidases I/II. Deglucosylation is essential for N-linked oligosaccharide maturation in the Golgi apparatus, but in the ER, sequential removal of glucose residues is critical for the progression of the calnexin/calreticulin cycle, a mechanism facilitating lectin-assisted folding of glycoproteins coupled with their quality control. Alternative deglucosylation allows glycoproteins to bypass the cycle and thus evade ER-localised quality control machinery. However, it is of interest to determine whether the deglucosylation of glycoproteins by endomannosidase is functionally linked to a post-ER quality control pathway. Previous studies have demonstrated that following glucosidase inhibition, misfolded glycoproteins are processed by endomannosidase and acquire the ability to become secreted. Hence, the processing of glycoproteins by endomannosidase was postulated to be non-selective, resulting in the progression of its products in the secretory pathway. To evaluate whether the described effect of endomannosidase is universal, we have empoyed a method of free oligosaccharides (FOS) analysis. FOS are released from glycoproteins prior to their proteasomal degradation as part of ERAD. By carrying out qualitative and quantitative analysis of FOS, we can assess the global levels of ERAD and probe the pathways of glycoprotein disposal. Our data show that following processing by endomannosidase, a fraction of glycoproteins is targeted back to the ER and is subjected to degradation, resulting in the production of deglucosylated FOS. We have therefore identified a novel ERAD pathway, leading us to speculate about the presence of a sorting mechanism for glycoproteins operating in a post-ER location.

Glyco(bio)technology I (Friday)

099: Biofunctionalization of biomaterial surfaces with glycan structures and human galectins

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The extracellular matrix (ECM) coordinates cellular responses like adhesion, proliferation and differentiation in vivo. Therefore the mimicry of this natural microenvironment on a biomaterial is a promising approach for tissue engineering and implantable surfaces. The ECM is composed of different glycostructures and ECM glycoproteins such as laminin and fibronectin. Galectins, β-galactoside binding proteins, are able to crosslink those structures and therefore promote cell-cell- and cell-matrix-interactions. Our ongoing work focuses on the formation of an artificial ECM as bioactive coating for biomaterial surfaces using glycans and galectins. The chemo-enzymatic synthesis and coupling of poly-N-acetyllactosamine (poly-LacNAc) mixtures onto biomaterial surfaces have been established in our group [1, 2]. Single glycan structures with defined chain length can be isolated out of the mixture by preparative reversed phase HPLC. Now we concentrate on the modification of isolated linear poly-LacNAc-structures to achieve specific binding affinities for selected galectins. Through specific glycan-galectin interactions cell adhesion and proliferation of different cell types might be regulated. Therefore, poly-LacNAc glycans were modified using *Dactylium dendroides* galactose oxidase, which was proven to oxidise selectively the terminal non-reducing galactose moiety. Conversion of C6-hydroxyl group to an aldehyde and subsequent pH dependent elimination of water under conditions of heat have been demonstrated by HPLC-ESI-MS and NMR analysis. The binding specificity of recombinant human galectin-1 and -3 to those modified and unmodified poly-LacNAc-structures is currently evaluated by enzyme linked lectin assays. Significantly varying affinities have been shown for the different glycans. In addition, an artificial extracellular matrix was created and analysed by crosslinking the glycoprotein standard asialofetuin or surface-bound poly-LacNAc to ECM-glycoproteins by galectins. Work is in progress to analyse the potential of different glycans and galectins as components of an artificial ECM for adhesion and proliferation of endothelial cells.

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100: The Nuclear Import of Nanoparticles by Displaying Glycoside Cluster on the Surface

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The nuclear pores located on the nuclear membrane work as a selective barrier to nuclear import, and the passage through the nuclear pores is an important technique in drug delivery systems (DDS). The transport of large cargo molecules (larger than ~40 kD) into the nucleus generally requires the aid of nuclear transport proteins, such as importins. They bind to nuclear localization signal (NLS) sequences in cargo molecules and transport these molecules into the nucleus through the nuclear pore. However, when the cargo molecules are negatively charged, such as DNA, the NLSs do not work efficiently. Therefore, neutral and biocompatible molecules are required to assist their nuclear transport. Previously, we have reported that maltotriose-displaying quantum dots (Glc3-QDs) entered into the nucleus from cytosol, whereas PEG-displaying QDs did not. The increasing of the surface density of Glc3 drastically accelerate the nuclear import (cluster glycoside effect). The increasing of the length of glucose unit also affected the efficacy of nucelar import. We expect that maltooligosaccharide may directly interact with nuclear pore. The aim of this study is to clarify the mechanism of the nuclear import of maltooligosaccharide-displaying QDs(maltooligo-QDs). Nucleoporin62, which is an internal protein in the nuclear pore, was expressed using *Escherichia coli*. Then, the kinetic parameters of the binding of maltooligo-QDs to nucleoporins62 were obtained using a surface plasmon resonance system. We found that maltooligo-QDs rapidly entered the cellular nucleus and had a high affinity to nucleoporin62 (Kd= 16nM), whereas PEG-QDs did not (Kd = 660nM). We concluded that this high affinity to nucleoporin62 is the driving force behind the nuclear import of maltooligo-QDs. We will discuss the effect of length of glucose unit, transport mechanism and efficiency.

101: Photo-click immobilization on polymeric surfaces – Versatile chemistry for the preparation of carbohydrate arrays

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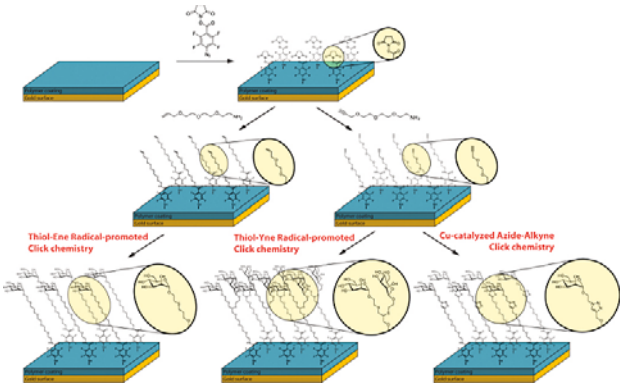
We have developed several methodologies for efficient carbohydrate attachment to polymeric surfaces for the use as analysis tools for protein-carbohydrate interactions. A specific bifunctional linker was designed for the immobilization method, efficiently connecting the polymeric substrate to the carbohydrates. The method utilizes efficient photocoupling of stabilized perfluorophenyl azides (PFPAs) to generate alkene-/alkyne-functionalized surfaces. The surfaces were subsequently reacted by either highly chemoselective Copper catalyzed Azide-Alkyne Cycloaddition or radical promoted thiol-ene/yne coupling. The methods enable rapid and convenient protocols to the general attachment of azide-/thiol-functionalized structures and were used to fabricate a range of surfaces presenting a variety of carbohydrate structures on different polymeric surfaces. The surfaces were evaluated in real-time studies using a QCM flow-through system with a series of different lectins.



102: Understanding and controlling O-GlcNAc processing enzymes in vitro and in vivo (Keynote)

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A common form of protein glycosylation in which serine and threonine residues of nuclear and cytoplasmic proteins are post-translationally modified with O-linked 2-acetamido-2-deoxy-β-D-glucopyranose residues (O-GlcNAc) is found in the nucleocytoplasm of multicellular eukaryotes. Unlike better known forms of glycosylation occuring within the secretory pathway, O-GlcNAc is a dynamic modification that is turned over more rapidly than the proteins that it ornaments. Dysregulation of O-GlcNAc has been implicated in the etiology of various diseases including type II diabetes and Alzheimer's. Two enzymes process O-GlcNAc; O-GlcNAc transferase (OGT) mediates installation of O-GlcNAc and O-GlcNAcase (OGA) catalyzes removal of O-GlcNAc from proteins. Here we describe research into understanding the chemical biology of these enzymes and the development of chemical tools for manipulating O-GlcNAc levels in tissues with a focus on recent results from the laboratory.

Glyco(bio)technology II (Friday)

103: Mushroom glycoengineering for the production of therapeutic glycoproteins

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The market for N-glycosylated therapeutic proteins represents multi-billion dollars in sales and is growing more than 5% each year. This requires cost-effective production platforms that display correct and homogeneous N-glycosylation. Previously, we investigated the N-glycosylation pattern of mushroom-forming fungi[1]. Based on the advantageous glycosylation profile observed, we proposed to use mushrooms (basidiomycetes) for the production of therapeutic proteins[2]. We have been studying the secretory pathway of basidiomycetes to be able to accomplish humanization of N-glycosylation. We show the first steps towards humanized N-glycosylation in basidiomycetes. We will elaborate on phenotypic effects and strain performance upon different glyco-engineering modifications and report on capacity of resulting strains to produce therapeutic glycoproteins.

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104: Humanization of the plant N-glycosylation pathway for the production of therapeutically relevant proteins

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Many therapeutically relevant proteins are N-glycosylated and need proper glycosylation for optimal efficacy. This is particularly true for monoclonal antibodies (mAbs), the fastest growing class of biopharmaceutical products. Thus glycoengineering has become a major focus in the biopharmaceutical industry. Plants serve as potential powerful expression system for the generation of therapeutically relevant proteins. As higher eukaryotes plants have the advantage that they carry out complex N-glycosylation. However shortcomings are the presence of plant specific glycan residues and the lack of sialylation. Here we present the modification of the N-glycosylation pathway in *N. benthamiana*, a tobacco related plant species widely used for recombinant protein production. Using knock down and knock in approaches we were able to generate *N. benthamiana* glycosylation mutants with human type glycosylation (Strasser et al., 2008, 2009). We show the generation of mAbs with different glycosylation profiles at great homogeneity and demonstrate the influence of Fc glycosylation to their functional activities. Plant-derived anti viral mAbs exhibit enhanced in vitro and in vivo neutralization potency when lacking core fucosylation, irrespective of its plant specific α1,3 or the human type α1,6 linkage. Finally, we show efficient in planta protein sialylation, the most complex type of human glycosylation. This required the reconstruction of the sialic-acid pathway and was achieved by the coordinated transient expression of six mammalian proteins in plants (Castilho et al., 2010). In summary we demonstrate the efficient generation of different protein glycoforms at great homogeneity. Our achievements pave the way not only for generating therapeutic glycoproteins with optimized biological activities obtained by a customized N-glycosylation profile but also for studying the impact of different glycoforms in biological processes.

105: Insect cells for antibody production: evaluation of an efficient alternative

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In recent years there has been an increase in both availability and demand for therapeutic monoclonal antibodies. Currently, most of these antibodies are produced by stably transfected mammalian cells. In this study we evaluated the use of different baculoviral insect cell systems as an alternative for commonly used production schemes. We expressed the human anti-gp41 antibody 3D6 in various insect cell lines and compared product yield, specificity and glycosylation patterns with a 3D6 antibody expressed in Chinese hamster ovary cells. We determined the N-linked oligosaccharide structures present on asparagine-297 in IgG_1 heavy chains and tested the functionality in terms of antigen binding and the ability to elicit effector functions. Antibodies expressed in all insect cell lines displayed highly specific antigen binding. In general, the insect-produced antibodies carried, as the CHO-produced form, fucosylated N-glycans, in some cases including high levels of core α1,3-fucose. This indicates that in all systems glycoengineering may be required in order to produce optimal glycoforms of this antibody.

106: Antibody engineering for developing next-generation therapeutic antibody; The importance of controlling post-translational modifications and its homogeneity as drugs (Keynote)

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Currently, over 27 recombinant monoclonal therapeutic antibodies have been approved since the late 1990s, and these agents represent a major new class of drugs that

confer great benefits not otherwise available to patients via small molecule drugs. However, several issues have been also emerging in antibody therapy, such as high cost due to the high doses requirement and insufficient efficacy against some obstinate diseases. Recent study have shown that human serum IgG strongly inhibits therapeutic antibody effector function of ADCC, which is closely related to the clinical efficacy of anticancer processes in humans in vivo. Unfortunately, currently-licensed therapeutic antibodies composed of a mixture of fucosylated and non-fucosylated IgG fail to achieve maximized ADCC. Clinical trials using non-fucosylated therapeutic antibodies with enhanced ADCC are underway, and have shown their remarkable physiological activities in humans in vivo. In this presentation, we will introduce the new platform of next-generation therapeutic antibodies, Potelligent®, generated by antibody Fc oligosaccharide engineering, based on the importance of post-translational modifications and its homogeneity as drugs.

Company of Biologists' Young Glycobiologists' Symposium

107: "Same Same But Different": N-Glycosylation in *Acanthamoeba*

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Numerous species of the protozoan *Acanthamoeba* are known for being opportunistic pathogens as well as representing carriers and reservoirs for pathogenic mycobacteria. As many host-pathogen interactions involve glycans that function as "communication tools" between the causative organism and the target host, knowing the N-glycomic repertoire of a pathogen can be helpful in deciphering its methods of establishing and sustaining a disease. Considering that an *Acanthamoeba* lectin is involved in binding to host cells, it is particularly of interest to study the pathogen's own N-glycome. For this study N-glycans of eight strains from five genotypes of *Acanthamoeba* were purified from trophozoite cells and compared using reverse phase and normal phase HPLC, MALDI-TOF MS and MS/MS. We were able to identify novel structures carrying pentoses and fucoses as well as describe the activity of an α-1,6 core-fucosyltransferase. The glycomic analysis of this human pathogen revealed, on the one hand, structures commonly found in eukaryotic cells such as oligomannose structures. What is setting *Acanthamoeba* apart from obligate parasites is its apparent capability of synthesizing the complete lipid linked oligosaccharide precursor dolichol-PP-$GlcNAc_2Man_9Glc_3$, which can be deduced from the presence of large oligomannose structures such as Man9. On the other hand, each strain also features novel N-glycans which are uniquely modified as compared to any N-glycan found to date. Based on this mix of familiar and unfamiliar structures the *Acanthamoeba* glycome can truly be described as being "same same but different". This work is supported by the FWF project number P20565.

108: Withdrawn

109: Withdrawn

110: Structure determination of bacterial mucus-binding proteins and their functional role in adhesion to host glycans

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The mucus layer covers the epithelial cells of the gastrointestinal (GI) tract and protects the underlying mucosa from the lumen content. The main structural components of mucus are highly glycosylated mucin proteins carrying mainly O-linked glycan chains characterised by a high level of structural complexity and diversity. Protein-carbohydrate interactions are believed to play an important role in the adhesion of resident gut bacteria to the mucus layer. However, the nature of the ligands and the specificity of the interaction remain to be elucidated. Our research focuses on lactobacilli mucus-binding proteins (MUB) whose presence on the bacterial cell-surface contributes to bacterial attachment to the protective mucus layer. MUBs are composed of tandemly-arranged mucus-binding amino acid sequence repeats (Mubs). The 353 kDa MUB from the *L. reuteri* strain ATCC 53608 consists of two types of repeats, Mub1 and Mub2, present in six and eight copies, respectively. We determined the first crystal structure of a type 2 Mub repeat (184 amino acids) at 1.8-Å, displaying high structural similarity to the repeat-unit of the *Peptostreptococcus magnus* Protein L (PpL), an immunoglobuling (Ig)-binding surface protein, and we showed that Mub repeats were able to interact with a number of mammalian Igs in vitro. Our current work is to obtain structural information on type 1 Mub repeats and multi-repeat modules to gain further insight into the structural organisation of MUB. In addition, we focus on the characterisation of Mub repeat interaction with mucins and mucin glycans using a range of biophysical methods including in vitro binding assays, carbohydrate arrays and Isothermal Titration Calorimetry. Elucidating the molecular basis of host-bacteria interaction

is crucial for the understanding of host colonisation and the beneficial role of lactobacilli in the GI tract.

111: Histone-code regulates brain specific expression of N-acetylglucosaminyltransferase-IX (GnT-IX)

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Tissue-specific expression of glycan is regulated by various factors including glycosyltransferases. Although most of glycosyltransferase genes have been cloned so far, the molecular mechanism underlying the tissue-specific expression of glycosyltransferase has been poorly understood. Recently, we cloned a novel brain-specific GlcNAc transferase, GnT-IX (Vb) (1), which exhibits branching activity toward O-mannose glycan (2). Here we examined the gene-regulation mechanism of this brain-specific glycosyltransferase.

First, we employed epigenetic approach because epigenetic mechanisms, such as DNA-methylation, histone modification or non-coding RNA, are fundamental for gene regulation. Using ChIP (chromatin immunoprecipitation) with antibodies against active and inactive chromatin histone marks, we revealed that chromatin activation occurs around GnT-IX core promoter in neural cell-specific manner. Moreover, forced chromatin activation by the treatment with histone deacetylase inhibitor induced GnT-IX even in non-neural 3T3-L1 cells, indicating that neural specific "histone code" actually regulates GnT-IX expression.

Next, we tried to identify pivotal transcription factors which trigger GnT-IX transcription in activated chromatin in neural cells. Using luciferase assay, gel-shift analysis and ChIP, we identified the most important cis-element and two transcription factors, CTCF and NeuroD1, which bound to and activated GnT-IX promoter. Depletion of these two factors by siRNA caused 50% reduction of GnT-IX expression, indicating that these factors actually regulated GnT-IX in vivo. Moreover, we showed that the binding of CTCF is highly dependent on chromatin activation state. These results indicate that CTCF- and NeuroD1-dependent epigenetic mechanism governs the brain-specific GnT-IX expression. Finally we showed that expression of other neural glycosyltransferases such as ST8Sia-IV and GlcAT-P is also regulated by neural specific histone code, suggesting that epigenetic chromatin activation is generally required for tissue-specific glycan expression.

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112: Biotoxicity of the fungal chimerolectin MOA

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The *Marasmius oreades* agglutinin (MOA) is a Galα1,3Gal/GalNAc specific lectin that was isolated from the fairy ring mushroom as a compound agglutinating blood group B erythrocytes. Structural studies previously revealed a dimeric organization of MOA with the protomer consisting of an N-terminal ricin B-type lectin domain and a C-terminal domain serving as dimerization interface. Latter domain adopts a novel fold that shows structural similarity to catalytically active proteins, suggesting that, in addition to its carbohydrate-binding activity, MOA has an enzymatic function. Here we demonstrate a carbohydrate-binding dependent toxicity of MOA towards the model nematode *Caenorhabditis elegans* and identify glycosphingolipids of the arthroseries as the ligands in the worm. We further show that MOA has cysteine protease activity and demonstrate a critical role of this catalytic function for MOA-mediated nematotoxicity. The proteolytic activity of MOA was dependent on calcium-binding and favoured by an alkaline environment, suggesting that these conditions act as trigger factors in activation of the toxin at the target location. Our results suggest that MOA is a fungal toxin similar to bacterial binary toxins possibly directed against predators such as fungivorous soil nematodes.

113: Incidence of O-GlcNAcylation on β-catenin oncogenic properties: O-GlcNAcylated β-catenin, a novel marker of early colorectal cancer?

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During the first steps of the colorectal cancerization, the Wnt/β-catenin pathway is the first to be modified. Indeed in such pathologies, an aberrant stabilization of the oncoprotein β-catenin, the key component of this pathway, is observed leading to a loss of the cell proliferation control. The regulation of the β-catenin properties is an essential point for cancer development comprehension. During this process, post-translational modifications (PTM) play a crucial role; for instance, the phosphorylation of the destruction box (D-Box) of β-catenin leads to its proteasomal degradation and accordingly controls its oncogenic properties. In our lab, we study the impact of another PTM, the O-GlcNAcylation, on the behaviours of β-catenin. This glycosylation is highly

dynamic and abundantly modifies cytosolic, nuclear and mitochondrial proteins. We have especially observed that O-GlcNAcylation levels are elevated in cells stemming from colorectal cancer in comparison with normal cells. We have shown that an increase of O-GlcNAcylation level protects β-catenin against degradation. We identified a major O-GlcNAcylation site inside the D-Box, siege of a Phosphorylation/O-GlcNAcylation antagonism that regulates its expression level. It is currently accepted that O-GlcNAcylation level is affected by extracellular glucose concentration. In this way, we demonstrated that the use of high-glucose media stabilizes β-catenin, promotes its transcriptional activity and activates the cell proliferation reflecting an increase of its oncogenic activity. More generally, increase of O-GlcNAcylation level promotes proliferative activity. This result is also correlated with an increased interaction β-catenin/OGT, the enzyme which adds GlcNAc residues onto proteins, during the cell cycle resumption. We propose that the O-GlcNAcylated β-catenin is a new selected candidate for the diagnosis of early colorectal cancer.

114: Applications of High-Throughput HPLC based N-Glycan Analysis for Biomarker Screening in Large Cohorts of Patients

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Glycosylation is the most complex post-translational protein modification. Over half of all proteins are glycosylated and to elucidate their function we also have to understand their glycan moieties. Recent advances in understanding the importance of glycosylation have placed renewed emphasis on glycan analysis. Our group is working on the development of technologies for structural N- and O-glycan analysis, with special emphasis on high-throughput methodologies towards biomarker discovery in cancer and other diseases. The first part of the presentation will discuss high-throughput HPLC method for structural determination and quantification of plasma N-link glycans. This method is advantageous in respect to other developed methods as it provides information about monosaccharide sequence, linkage, and arm-specificity for charged and neutral glycans. This strategy can be used for analysis of large cohorts of samples in relatively short time, thereby offering relatively a straightforward, quick and cost effective option. The second part of this presentation will highlight applications for the HT-HPLC technique. A demonstration of how this faster method can facilitate screening for biomarkers in a large number of patients will be presented. An example is the investigation of plasma samples from 1458 residents of the Orkney Islands to understand the complex genetic regulation of glycosylation and link N-glycan distributions to environmental, heritable and disease factors. Another application involves screening of Galactosemic patients in regards to specific galactosylation of IgG as a biomarker of galactose tolerance. High-throughput glycome studies are promising tools to address the challenges of integrating various systems and coupling glycomics with proteomics, genomics, metabolomics and environmental determinants.

115: Sweet protection: innate immunity conferred by salivary sugar antigens?

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Mucosal epithelial surfaces, such as those that coat the oral cavity, are common sites of microbial colonization. These interactions involve adherence between the glycans of the host and the carbohydrate binding proteins of the pathogen. We show that glycoproteins in the saliva that constantly bathes the buccal cells of the epithelial surface in the mouth are involved in an innate protective mechanism. In particular, that the glycans released from these salivary glycoproteins inhibit the interaction of the oral pathogen *Candida albicans* with buccal epithelial cells.

A flow cytometry based binding assay was developed to quantify the interaction between buccal cells and the oral pathogen *C. albicans*. N- and O- linked protein glycosylation of saliva and buccal cell membranes was characterised using capillary carbon LC-ESI-MS/MS. Characterising the surface sugars of buccal cell mucosa defines the potential glycan epitopes that are available to bind with pathogens in the oral cavity. Therefore glycan analogs of the carbohydrate receptors involved in the interaction can be the basis for carbohydrate based anti-adhesive drugs. The inhibitory potential of saliva, and of the glycans released from the salivary glycoproteins was assessed using the binding assay.

The glycans released from buccal cell membranes were found to be very similar qualitatively, but differed quantitatively, from the glycans released from salivary proteins. MS signal quantification of terminal glycan epitopes such as Lewis, sialylated Lewis, ABH and other glycans from whole saliva and buccal cell membranes, thus have a related expression that can serve to competitively inhibit pathogen interaction with host surfaces. We also show that individuals' glycans on buccal epithelial cell membrane proteins and whole saliva differ mainly with respect to their blood group antigens reflecting their ABH, Lewis and secretor status. The role played by sugar structural epitopes in the interactions of *C. albicans* with buccal epithelial cells and saliva in the oral cavity of various individuals will be described.

116: Introduction of tetra-antennary N-glycans in *Nicotiana benthamiana* plants

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Until now pharmaceutical proteins were mainly produced in bacteria, yeasts or mammalian cells. Plant systems can offer advantages for cost effective and flexible production of biopharmaceuticals. For this, the magnICON expression system was developed which enables high level production of biotherapeutics in leaves of *Nicotiana benthamiana* plants. This system allows generating protein levels of 300 mg monoclonal antibodies per kg fresh weight within 7-9 days.

Although protein synthesis is conserved between plants and humans, there are differences in the N-glycosylation pathway. Since these differences can influence the safety and efficacy of plant made pharmaceuticals (PMPs) the plant N-glycosylation pathway needs humanization. Although many of the differences have already been addressed, little attention has been given to the structural differences. Our research is aimed at introducing multi-antennary glycan structures in *N. benthamiana* by expressing the responsible enzymes GnT-IV and GnT-V. In addition, this humanisation step will be combined with the removal of xylose and fucose (XylT/FucT RNAi plants) and the addition of galactose.

For the production of multi-antennary N-glycans the non-host glycosyltransferases, GnT-IV and –V, were fused to the plant specific localization signals of XylT and FucT. Both wild type and XylT/FucT RNAi plants were used for transformation. The levels of multi-antennary glycans were compared in different mutant backgrounds and with different plant-specific Golgi localization signals. The plants with the highest relative amount of multi-antennary N-glycan structures were used to transiently express a therapeutically relevant protein, such as EPO, with the magnICON system.

The presented research shows that it is possible to introduce humanised, multi-antennary N-glycan structures in plants and combine this modification with the removal of xylose and fucose residues. Furthermore, it was shown that a recombinant protein expressed in these plants carried the humanised N-glycan structures. This opens the road to further expand the PMP production platform by introducing galactose and sialic acid residues and create fully humanised plants.

Euroglycoscience Forum Glycoimmunology Workshop (Tuesday)

117: The T cell balance during chronic helminth infections

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Helminth infections are the strongest natural stimuli for type 2 immune responses, characterized by elevated IL-4, IL-5 and IL-13 along with high levels of IgE and IgG4. Using flow cytometry, the number of T cells expressing Th2 cytokines or the transcription factors that indicate commitment to Th2 were shown to be elevated in helminth infected subjects. In addition, chronic helminth infections are associated with T cell hyporesponsiveness which might be due to the presence of regulatory T cells (Treg). Indeed in several studies the numbers of $CD4^+CD25^+Foxp3^+$ cells has been shown to be elevated in helminth infected subjects. These cells are able to down regulate effector T cells and thereby can play a role in preventing their expulsion from their human host and also to keep immunopathological reactions to a minimum. In support of a beneficial effect of regulatory T cells, recently it was shown that the balance between Treg and Th17 might be distroted in a helminth infected subject exhibiting clinical pathology. However, elevated regulatory cells might have a negative effect on responses to vaccines. Indeed in some studies the presence of helminth infections has been associated with poor T cell responses to vaccination. A full understanding of the T cell responses during chronic helminth infections might help the design of new measures to control infection and pathology.

118: Pattern recognition and anti-microbial immunity: The role of C-type lectins

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The innate ability to detect pathogens is essential for multicellular existence, and has been achieved through the evolution of germ-line encoded receptors which can recognise non-self structures, the so-called “pattern recognition receptors” (PRR). One such receptor is Dectin-1, a type II transmembrane glycoprotein with a single extracellular non-classical C-type carbohydrate recognition domain (CRD) and a cytoplasmic tail possessing an immunoreceptor tyrosine-based activation-like (ITAM) motif. Dectin-1 is predominantly expressed on myeloid cells and recognises β(1→3)-linked glucans. We and others have demonstrated that this receptor mediates a variety of cellular responses to β-glucans, including phagocytosis, endocytosis and the oxidative burst and can induce the production of arachidonic acid

and numerous cytokines and chemokines. These responses are triggered through the cytoplasmic ITAM-like motif of this receptor, utilising novel signalling pathways involving a unique interaction with Syk kinase and collaborative signalling with the TLRs. Dectin-1 is the first example of a signalling non-Toll-like pattern recognition receptor being involved in the induction of protective immune responses, and through these activities Dectin-1 plays a fundamental role in anti-fungal immunity in mice and humans. Here, we will present more recent data on the role of this and other C-type lectin receptors in antimicrobial immunity.

119: Induction of Antigen-Specific B cell Tolerance through the Development of a Versatile Platform to Target Siglecs.

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Sialic acid binding Ig-like lectins (Siglecs) are a family of carbohydrate surface receptors that are predominantly expressed on cells of the immune system. CD22 and SiglecG are two members of the Siglec family that are expressed on B lymphocytes and are proposed to be negative regulators of B cell receptor signaling through their ability to recognize sialic acid-containing glycans. Recent studies have shown that multivalent presentation of high affinity CD22 and SiglecG ligands in cis with a T-independent antigen can tolerize B cells (Duong et al., J. Exp. Med., 2010). Here we describe studies aimed at showing the generality of this phenomenon. To realize this aim, a novel liposomal nanoparticle platform was developed for presentation of both high affinity Siglec ligands and antigen. Chemical conjugation of the Siglec ligand and antigen to this platform is facile and, importantly, this platform is highly compatible with in vivo studies. Using this new platform, robust B cell tolerance is achieved toward both T-independent and T-dependent (protein) antigens in mice. Mechanistic studies provide clear evidence that: i) CD22 and SiglecG are the targets; ii) apoptosis of B cells is the mode of tolerance; iii) tolerization is highly selective to B cells containing a BCR that is specific for the targeted antigen. These studies reinforce the hypothesis that CD22 and SiglecG are critical components of a B cell that allows it to distinguish self from non-self. This method has clear potential for treatment of autoimmune diseases and allergies by antigen specific tolerization of B cells. (NIH GM060938 and AI050143 and HFSP Fellowship LT001099/2010-L)

120: Regulation of B cell activation by inhibitory receptors of the Siglec family

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CD22 (Siglec-2) and Siglec-G are inhibitory co-receptors of the Siglec family which inhibit BCR-induced Ca2+ signalling in B cells. While CD22 inhibits responses in all B cells, Siglec-G acts specifically on B1 cells. CD22 binds to α2,6 linked sialic acids which occur in cis on the B cell surface. We addressed the question how this ligand binding in cis affects the inhibitory function of CD22 by generation of two novel knockin mice with mutated ligand binding or mutated signalling domains of CD22 and found reciprocal regulation of Ca2+ signalling by these domains. Siglec-G-deficient mice showed a large expansion of B1 cell numbers, which is a subpopulation of B cells in the mouse. This highly increased B1 cell compartment is due to increased survival and altered selection of Siglec-G-/- B1 cells. We examined a possible redundancy of Siglec-G and CD22 by establishing Siglec-G x CD22 double-deficient mice. These mice develop spontaneous autoimmunity, which was not observed in single KO mice. This shows that both Siglecs together control B cell tolerance and contribute to prevent spontaneous systemic autoimmunity.

121: Targeting C-type lectin receptors with synthetic carbohydrates to modulate immune responses

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In the immune system, there are different classes of lectins recognizing conserved carbohydrate structures of glycoproteins or glycolipids. C-type lectin receptors (CLRs) represent a large family expressed by antigen-presenting cells (APCs) such as dendritic cells. CLRs bind to carbohydrate structures on the surface of pathogens and thus are essential for the initiation of an immune response. In addition, some CLRs contribute to homeostasis of the immune system. Targeting CLRs is an attractive approach to deliver drugs into APCs or to modulate APC function. Initial results of our laboratory demonstrate that synthetic glycans can be utilized for cell-specific drug delivery as well as targeting of CLRs on APCs. To identify yet unknown carbohydrate ligands of CLRs, CLR-Ig fusion proteins consisting of the carbohydrate-binding domains of CLEC-1, CLEC-2, DCIR, DCAR, MCL, Mincle, Dectin-1 and -2, Lox-1, CLEC-9a, MICL, CLEC-12b, MGL and the constant F_c fragment of human immunoglobulins were generated through cloning and eukaryotic expression. The glycan array platform was used for screening of carbohydrate-lectin interactions between the CLR-Ig fusion proteins and amine- and thiol-functionalized synthetic oligosaccharides such as high-mannose structures, phosphatidylinositol mannosides, heparins, lipoarabinomannans and sialylated oligosaccharides. Novel carbohydrate ligands were identified illustrating the potential of the glycan array technology for immunological studies. Carbohydrate ligands of CLRs were chemically coupled to model

antigens to investigate the effect of a CLR engagement on immune stimulation. In proof-of-principle experiments, uptake of antigens by APCs as well as antigen processing and presentation to T cells were markedly increased. In conclusion, CLRs can be used for specific targeting of APCs, in which CLR ligands are used as adjuvants to elicit CLR signaling.

122: Is it possible to correct the anergy of human tumor-infiltrating lymphocytes?

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We have observed that human TIL show impaired IFN-γ secretion, apparently due to a reduced mobility of T cell receptors trapped in a lattice of glycoproteins clustered by extracellular galectin-3, a lectin secreted by tumors and macrophages. A short treatment with an anti-galectin-3 antibody or N-acetyllactosamine, a galectin ligand, restored IFN-γ secretion. Galectin competitor ligands, e.g. LacNAc, are rapidly eliminated in urine, preventing their use in vivo. We recently found that a plant-derived polysaccharide, DAVANAT, a galactomannan approved for human clinical trials, boosted the ability of TIL to secrete cytokines upon ex vivo stimulation, and increased their cytotoxicity. CD8 TIL populations, which were isolated from ovarian carcinoma ascites and treated with DAVANAT, can lyse autologous tumor cells. This treatment had also an effect on CD4 TIL but not on CD8 or CD4 blood T cells. We are currently screening various other galactomannans for their effect on human TIL. We are also testing a number of polysaccharides extracted from plants and fungi that have been reported to have immunostimulatory capacities. Our previous results in a mice model suggested that a combination of a galectin ligand and therapeutic vaccination may induce more tumor regressions in cancer patients than vaccination alone. We will therefore launch a clinical trial combining peptide vaccination and injections of DAVANAT.

Euroglycoscience Forum Methods in Glycolipid Analysis Workshop (Tuesday)

123: Glycosphingolipid derivatizations for mass spectrometry and microarray "omics" applications

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The range of functional carbohydrate binding protein (CBP)-carbohydrate ligand recognition events covers the entire spectrum from endogenous intra- and intercellular interactions mediating embryonic development and tissue differentiation to interspecies interactions such as infection and defense. Powerful examples of the latter are bacterial adhesins, which exploit adventitious binding sites on the surfaces of target host cells as an initial step for colonization. Mammalian cell surface glycosphingolipids (GSLs), for example, are preferred targets for adhesins of a variety of bacterial species. Glycan arrays are accepted as important tools for making sense of the enormous complexity of CBP–ligand interactions, but their usefulness ultimately depends on the availability of reliable, detailed knowledge of the structures of glycans incorporated into them, regardless of whether these are synthetic or isolated from natural sources. This talk will focus on (i) methods for characterization of GSL glycan structures and GSL expression profiles (GSLomics) based on ion trap (or Orbitrap) MS^n techniques, and (ii) applications of novel GSL derivatives facilitating their isolation and characterization and/or their incorporation into microarrays. Generation of these derivatives depends on removal of the GSL fatty-N-acyl moiety by an enzyme, sphingolipid ceramide-N-deacylase (SCDase). Following this, the free sphingoid 2-amino group becomes available for reaction with any of a wide variety of useful amine-selective derivatizing reagents. Although a number of degradative reactions have been used successfuly for generating reactive GSL intermediates, an advantage of SCDase is that it doesn't depend on harsh chemistries or the presence of a sphingoid E4-unsaturation to function; nor does it, like ceramide gycanases, depend on the presence of a direct glycosyl linkage to ceramide. It can therefore be applied to a wider range of GSLs, including glycosylinositol phosphorylceramides from plants and fungi. A disadvantage is that information about N-acyl structure is lost. This still leaves a wide variety of applications for which this methodology presents an attractive alternative.

124: Mass Spectrometry-Based Characterization of Composition and Structure of Gangliosides as Human Brain Biomarkers in Health and Disease

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Comparing to mammalian non-neural cell types, brain cells contain the highest concentrations and the most complex compositions of sialylated glycosphingolipids – gangliosides. Gangliosides are primarily plasma-membrane components, enriched in membrane microdomains, and expressed, by their carbohydrate moieties, as cell surface antigens. They participate in vital cellular processes: cell-

to-cellcommunication,cellsignaling,growth,differentiation, apoptosis etc. Their composition is species- and cell type-specific. In human brain, distribution of gangliosides is neuroanatomical region-specific; the patterns specifically change during brain development, maturation and aging, while characteristic aberrations of the normal patterns occur duetodiseases(e.g.malignantalteration,neurodegeneration). Gangliosides are, therefore, recognized as valuable neuro-developmental markers and screened as potential diagnostic markers of diseases or therapeutic targets. Also, their usage as therapeutic agents has been under clinical trials.

Our groups continuously participate in characterizing biomarker ganglioside compositions and individual structures of defined human brain regions during development and aging, as well as of the histopatologically defined brain tumors. To collect detailed systematic and comparative data about ganglioside compositions including identification of novel structures we have implemented and optimized sensitive mass spectrometric (MS) approaches allowing ganglioside screening and sequencing directly from native complex mixtures of total gangliosides extracted and purified from tissue samples. The following MS systems have been used: capillary- or fully automated chip-based nanoelectrospray ionization interfaced to high-performance tandem mass spectrometers - a high-capacity ion trap MS, quadrupole time-of-flight MS, and Fourier-transform ion cyclotron resonance MS. Employing these MS approaches in complementation with chromatographic and immunochemical methods, we have documented that specific ganglioside patterns contain much larger number of individual species than previously reported. In this contribution, analytical potential of the mentioned strategies will be shown. Results will be presented demonstrating complexity of data collected by applying these strategies to characterize total ganglioside mixtures from selected regions of fetal/adult brain and brain tumors (glioblastoma, astrocytoma, meningioma, haemangioma, adenocarcinoma brain metastasis).

125: New Approach for Glycolipidomics -Molecular Scanning of Human Brain Gangliosides by TLC-Blot and MALDI-TOF MS-

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We have developed TLC-Blot system(Far-Eastern Blot) which made possible direct analysis of the transferred glycosphingolipids on PVDF membrane from High-performance TLC(HPTLC)-plate by immunological staining, chemical staining, enzymatic treatment and mass spectrometric (MS) procedure. An ion trap type matrix-assisted laser disorption/ionization (MALDI)-TOF (time of flight) MS equipment improved not only the molecular identification but also analysis of molecular species of lipids on the PVDF membrane. A new approach for glyco and lipidomics, molecular scanning technology by a combination of TLC-Blot and MALDI-TOF MS is developed and applied for human brain gangliosides separated from tissues with neural diseases and control patients. The analytical results clearly showed a change of ganglioside composition together with individual ganglioside molecular species especially in Alzheimer's disease. The results strongly suggested a large involvement of metabolic change of gangliosides in the progress of the disease. The present technology with molecular scanning on TLC-blotted membrane will provide valuable information to elucidate the significance of molecular species in neuronal functions such as neural transmission, memory, and learning.

126: Complementary triad of thin-layer chromatography, overlay technique and mass spectrometry as a versatile tool for exploring glycosphingolipid-based host-pathogen interaction

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Glycosphingolipid (GSL) analysis recently underwent a rapid expansion owing to the development of novel mass spectrometry (MS) methodologies that act as the key technique to identify structural and functional aspects of GSLs. A large body of studies have explored GSL-pathogen interactions as the initial event in infectious diseases. MS-based strategies have been developed aimed at characterizing GSLs of a certain cell type, organ or tissue and identifying specific targets of pathogens and pathogen-derived toxins. The heterogeneity of GSLs is mostly caused by variable oligosaccharides that vary in the number and sequence of monosaccharides and their anomeric configuration as well as linkage type. Furthermore, the ceramide variability, originating from the diversity of the long-chain amino alcohol and the fatty acid moieties, which both may vary in chain length and degree of unsaturation, further contributes to the large number of different GSL species. In view of this heterogeneity, a single analytical method would typically yield insufficient data, providing only partial structural information of individual GSLs. Substantial improvement of this situation is achieved by a versatile triad system that matches three complementary methods namely (1) silica gel-based TLC separation of GSLs, (2) their overlay detection on the TLC plate with oligosaccharide-specific proteins, and (3) direct and indirect MS-based structural characterization. MS analysis can be performed "on line",

i.e. directly from the TLC plate employing infrared matrix-assisted laser desorption/ionization (IR-MALDI) or "off line" from silica gel extracts from GSL bands using electrospray ionization (ESI) MS. Here we will present novel data on recent advances employing this triad system and address promising improvements in GSL receptor characterization using antibodies, Shiga toxins, bacteria and influenza viruses by means of direct IR-MALDI as well as indirect ESI MS for analysis of GSLs. This combinatorial approach represents a step forward in GSL receptor research aimed at the identification of GSLs involved in host-pathogen interaction and countering infectious diseases.

127: Isomeric analysis of oligomannosidic N-glycans and dolichol-linked precursors by PGC-LC-ESI-MS

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Oligomannosidic N-glycans and glucosylated precursors occur in a variety of isomeric forms, whose separate determination by two-dimensional HPLC, NMR or multi-stage MS has hitherto been a difficult task. The emerging roles of different precursor oligosaccharides in ER-quality control and ER-degradation pathways, the structural changes of oligomannosidic glycans in certain congenital disorders of glycosylation (CDGS 1c) or the fact that oligomannosidic glycans on HIV antigens can form immunogenic epitopes point at the need for a fast and sensitive means of distingiushing the oligomannosidic isomers. We have investigated the capacity of liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) with porous graphitic carbon columns for the determination of oligomannosidic isomers. Protein-linked N-glycans were released enzymatically and subjected to LC-MS analysis after reduction with borohydride and purificiation with carbon cartridges. Lipid-linked glycans were released from microsome preparations by mild acid hydrolysis thus bypassing biphasic partioning. Comparison with well characterized samples such as N-glycans from kidney bean or ribonuclease B, parallel analysis as pyridylaminated glycans by amide-silica and reversed-phase HPLC (2D-HPLC), application of α-mannosidases with certain specificities and work with ALG mutant plants led to the assignment of the relative retention times of essentially all of the isomers occurring during the degradation of the $Glc_3Man_9GlcNAc_2$ precursor oligosaccharide to $Man_5GlcNAc_2$ and beyond. The amazing selectivity of graphitic carbon allows for the complete structural assignment of oligomannosidic glycans in one chromatographic run with simple MS detection.

Euroglycoscience Forum Glycobioinformatics Workshop (Tuesday)

128: GlycoBioinformatics

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In contrast to other major Omics such as genomic and proteomic, glycomics lacks accessible, curated and comprehensive data collections that summarize the structure, characteristics, biological origin and potential function of glycans that have been experimentally verified and reported in the literature. This lack of glycan databases is one of the biggest hindrance to glycobiology research. Additionally, the sparseness of glycan databases hampers the development of bioinformatics tools for the interpretation of experimental data and the automatic determination of the glycan structure, therefore limiting the possibility of large scale high throughput glycomics studies. This presentation will provide a overview of the current status of the field and possible future developments and will set the scene for the following workshop.

129: UniCarb-DB: Functional assignment using epitope substructures

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As part of an ongoing initiative to improve and excel in the realm of glycomics, a consortium of experts has initiated the UniCarbKB knowledgebase. This platform will serve as the first point of reference for researchers, both experienced and those new to the field, when confronted with glycobiological questions. This knowledgebase includes the UniCarbDB database of experimentally annotated glycan structures (www.UniCarb-DB.org), which will be presented here. The usefulness of the database is due to the inclusion of experimental data to back up the structure assignments. This experimental evidence is either LC/MS and MS/MS and/or HPLC retention data. The database is thus an important analytical tool that will be a welcome addition to any workflow to aid the correct assignment of glycan structures. The first stage of pathogen attack e.g. *Campylobacter jejuni*, *Helicobacter pylori* etc. on host organisms can often involve binding to

one or many sugar sub-structures found on mucin type glycoproteins which line the protective epithelial barriers. The annotation of the complex glycan structures that contain these epitopes is one step closer to identifying the function of oligosaccharides in host-pathogen interactions as well as other disease states. We shall present the novel feature in UniCarbDB of structural epitope annotation. Stored glycan structures have been annotated using an internal dictionary/ontology of 163 different glycan epitopes compiled from a variety of glyco-conjugates. This powerful feature will allow for functional investigation of glycan structures.

130: Building Blocks for a New Glycomics Knowledgebase

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UniCarbKB is a new collaboration bringing together glycobiology researchers and bioinformatic developers to create a universal glycomics knowledgebase built on collections of experimental, structural and functional data. The creation of an information rich central hub will provide a crucial resource for glycobiology and glycomics research that will enhance the understanding and biological importance of glycosylation. A vital component of UniCarbKB is the implementation of a bioinformatics framework to support strategies that are generating overwhelming volumes of complex data. The workshop introduces UniCarb-DB (www.unicarb-db.org), a project that builds on and expands the success and features of EUROCarbDB. A major aim of UniCarb-DB has been to develop a resource for storing LC-MS and MS/MS data collections that will serve as a base for new analytical tools for data querying and spectra interpretation. We shall discuss a platform for storing oligosaccharide structures and fragment data characterised by these strategies. This will include a review of annotated O-linked data sets published by participating members built on those best practices developed by existing glycomics databases.

During the session we will present an overview for the requirement of a centralised curated glycan structure resource. We shall introduce recent efforts to develop this resource, with an emphasis on the incorporation of data from the publically available GlycoSuiteDB (www.glycosuitedb.expasy.org) into the UniCarb-DB framework. We shall also demonstrate how we have maintained the functions and tools developed previously by EUROCarbDB to create a robust bioinformatics solution to cater for future growth.

131: GlycoWorkbench / Glyco-Peakfinder: tools for the computer-assisted annotation of mass spectra of glycans

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Mass spectrometry is the main analytical technique currently used to probe the repertoire of glycan structures found in both complex mixtures (Glycomics) and attached to specific proteins (Glycoproteomics). The determination of glycan structures from the analysis of mass spectra is a laborious and error-prone task. GlycoWorkbench (Ceroni et al. 2008) and Glyco-Peakfinder (Maass et al. 2007) are two tools that take complementary approaches to guiding glycobiologists to the correct structure determination. This workshop will demonstrate the use of these tools in analyzing both MS and MS^n datasets, as well as generating spectrum annotations suitable for publication. An update will also be given on the ongoing work that is being carried to integrate the GlycoWorkbench/Glyco-Peakfinder workflow into the data submission pathway of the UniCarb-DB/EUROCarbDB. Ceroni, A. et al., 2008. GlycoWorkbench: a tool for the computer-assisted annotation of mass spectra of glycans. Journal of Proteome Research, 7(4), p.1650-1659. Maass, K. et al., 2007. “Glyco-peakfinder”- de novo composition analysis of glycoconjugates. Proteomics, 7(24), p.4435-4444.

132: GlycoBase and GlycoExtractor: tools for HPLC-based glycan analysis

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To support our high-throughput HPLC analysis, which provides detailed structural information for charged and neutral glycans, a range of bioinformatic tools has recently been developed in NIBRT. Here, we discuss two of them, GlycoBase and GlycoExtractor. The first version of the GlycoBase relational database, which contains the elution positions for 2-aminobenzamide-labeled (2AB) carbohydrate structures together with predicted products of their exoglycosidase digestions, was first published in 2008. Since this first variant of the database was presented to the scientific community, GlycoBase has undergone a number of changes. The publicly available variant of the database contains the normalised values of HPLC retention times for a limited number of carbohydrates. Later on, this version of the database was integrated into the publicly available EUROCarbDB framework including a number of its substructural search options. NIBRT’s proprietary variant of the database is under development to include many

improvements. The latest changes included the addition of normalised retention times for a number of glycan structures obtained by other methods, such as UPLC and Capillary electrophoresis, and improvements to the AutoGU tool, which assists the user in their data interpretation. Also, the number of O-linked carbohydrate structures has been significantly increased. GlycoExtractor is a web-based tool which was developed to automate the routine process of data extraction and to allow visualisation of the data. The first variant of the tool was presented to the scientific community in 2010 to initiate the creation of new data interchange resources for HPLC-glycan technologies, which can be easily integrated with different systems and software used in high-throughput HPLC data analysis. To improve GlycoExtractor's functionality, its second edition is being developed using the commercially available Waters Empower™ toolkit.

133: An update on SAGS, the Structural Assessment of Glycosylation Sites Database.

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Bioinformatics can bring an overall view on N-glycan occurrence in proteins which allow mapping relevant propensities at all structural levels. The Structural Assessment of Glycosylation Sites Database contains information on glycosydic linkages, glycan-protein linkages and on properties of the protein core around glycosylation sites such as: primary structure, secondary structure, geometry and composition of the protein surface around glycosilation sites, or glycan-protein contacts. SAGS contains also modules of statistical analysis of the data and its update is automated. During the last five years the volume of the database increased from 575 to over 2000 crystal structures containing N-glycosylated polypeptides, of which over 500 are non-redundant. These document the structure of 2150 different sequons of which around 1400 are occupied. Statistical analysis performed on SAGS indicate the existence of a number of structural patterns that prove useful in prediction, modeling and understanding a number of aspects related to occupancy, folding and oligosaccharide function in glycoproteins. For example, significant deviations in the expected amino acid composition were seen around occupied asparagines. Glycosylation alters the Asn side chain torsion angle distribution and reduces its flexibility. There is an elevated probability of finding glycosylation sites in regions where the secondary structure changes. The protein surface geometry around glycosylation sites is very diverse and needs an 11-classes taxonomy in order to describe it properly. However, a surprisingly large number of glycosylated asparagines have a low accessibility. These patterns may have significant implications for control of sequon occupancy; the evolutionary selection of glycosylation sites; the mechanisms of protein fold stabilization and the regional quality control of protein folding. Hydrophobic protein–glycan interactions and the low accessibility of glycosylation sites in folded proteins are common features and may be critical in mediating these functions.

Euroglycoscience Forum Glycoproteomics Workshop (Tuesday)

134: ETD-MS and mucin-type O-glycopeptides: getting closer to the holy grail?

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Changes in mucin-like domain glycosylation are seen in many diseases, particularly in cancer, but these domains tend to be very heterogeneous and heavily O-glycosylated, which makes them difficult to analyse by conventional means. Efficient site-specific characterisation of mucin-type glycopeptides containing high-density O-linked glycan clusters remains the most difficult aspect of glycopeptide characterisation and different strategies have been tried over the years to enable this information to be obtained. In addition to the technical challenges associated with the generation and isolation of appropriate glycopeptides from mucins containing tandem repeats, structural characterisation is extremely challenging due to i) glycan micro- and macro- heterogeneity at each site, ii) lack of a consensus sequence for O-glycosylation, iii) high density areas of occupied glycosylation sites, and iv) lability of some of the O-linked glycan moieties. We have used electron transfer dissociation (ETD) ion trap ESI-MS/MS to characterise both native and synthesised, densely glycosylated mucin-type O-linked glycopeptides containing typical O-linked glycans (i.e. mannose, Tn, T, acetylated T, and sialyl T) with various degrees of glycan occupancy, as a first step towards enabling these regions to be analysed routinely.

135: How glycopeptides (and peptides) behave on reversed-phase and graphitic carbon columns

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Direct analysis by LC-ESI-MS of the mixture of peptides and glycopeptides generated by proteolytic digestion allows the site-specific and quantitative glycosylation analysis of microgram quantities of a glycoprotein. On reversed-phase columns, the retention behaviour is essentially determined by the peptide moiety so that all glycoforms of a given peptide elute as one peak, albeit with shifted maxima. However, some RP columns -

without explicit mentioning by the supplier - exhibit ion-exchange properties, which complicates perception of the site-specific glycan pattern. Porous graphitic carbon binds free oligosaccharides and hence the glycan portion contributes considerably to glycopeptide retention. This poses an interesting means of isolating certain glycoforms or of determining if certain glycoforms found by RP-HPLC are actually existing entities or just in-source fragmentation artefacts. The influence of certain structural features of the glycan as well as of the peptide moieties will be discussed with some emphasis on antibody Fc glycosylation.

136: Characterisation of O-glycan structures and glycosylation sites of proteins in proteomic samples

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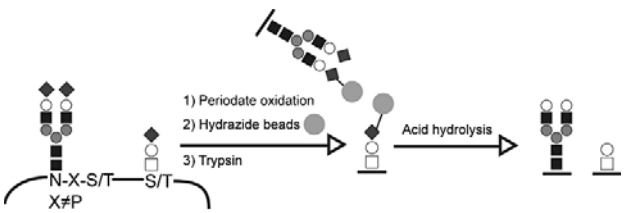
Many extracellular proteins are glycosylated with sialic acid containing glycans on Asn (N-glycans) and Ser/Thr (O-glycans). By analysing tryptic glycopeptides originating from such glycoproteins it is possible to identify both the glycan structures and glycosylation sites of proteins. We have developed a sialic acid capture-and-release method to enrich tryptic glycopeptides from the vast majority of unglycosylated peptides in tryptic digests (1). We used liquid chromatography/tandem mass spectrometry (LC-ESI-MS^n) for the analysis and managed to identify 36 N- and 44 O-glycosylation sites on glycoproteins from human cerebrospinal fluid (CSF). To specifically study O-glycopeptides we PNGase F treated CSF samples to remove interfering N-glycopeptides and were able to identify 32 additional O-glycosylation sites of which most were previously unknown.



We use collision-induced dissociation (CID) for glycan fragmentation, which is used to analyse glycan structures in the MS^2 and MS^3 spectra. CID-MS^3 of deglycosylated peptide ions from the MS^2 step results in peptide backbone fragmentation and is used for peptide (and protein) identification. In addition, we use electron capture/transfer dissociation (ECD/ETD) on FT-ICR and Orbitrap instruments, respectively, for peptide fragmentation in the presence of intact O-glycans in order to pinpoint the glycan attachment sites of O-glycopeptides containing several Ser/Thr residues. I will demonstrate step-by-step procedures to enrich and to analyse O-glycopeptides from proteomic samples. This includes the use of CID, ECD, ETD and HCD fragmentation techniques for both manual and automated O-glycopeptide characterisations.

Reference: (1) Nilsson J, Rüetschi U, Halim A, Hesse C, Carlsohn E, Brinkmalm G, Larson G, Enrichment of glycopeptides for glycan structure and attachment site identification, Nature Methods, 6, 809-811 (2009).

137: Bioinformatics in Glycoproteomics – Challenges, Frontiers and Solutions

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The development of suitable bioinformatic tools has been a crucial step towards the success of proteomics workflows in the last decade. Software and workflows that allow for automated and reliable identification of proteins from complex mixtures have become familiar companions in proteomics laboratories and the challenges of protein quantitation have also been addressed in a variety of ways. These developments provide scientists with a selection of tools and workflows to choose the most suitable one for their particular research. The inclusion of complex post translational protein modifications such as glycosylation into current automated workflows, however, still poses huge challenges for traditional protein identification algorithms. Different publically as well as commercially available glycoproteomics software tools have been described recently. This part of the glycoproteomics workshop is intended to present an overview of different currently available glycobioinformatic solutions and will discuss their benefits and limitations.

Euroglycoscience Forum Mass Spectrometry Workshop (Wednesday)

138: Molecular weight determination by mass spectrometry – the first step in the characterization of polysaccharides and glycoproteins

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An array of mass spectrometric techniques play since the introduction of the first, so-called "soft ionization" techniques (fast atom bombardment, liquid secondary ion mass spectrometry and Cf-252 plasma desorption) in combination with analyzer as linear time-of-flight, reflector, ion mobility, quadrupole or ion trap devices, a growing role in the characterization of oligosaccharides, polysaccharides and glycoproteins, particular due its unsurpassed sensitivity. The determination of the exact molecular mass and heterogeneity

as first step in the structural characterization without mass spectrometry is not anymore *state-of-the-art*. Even in case of secondary, tertiary and quaternary structure establishment of glycoproteins this technology array plays an increasing role.

The content of the brief workshop lecture will be divided into four main sections: I. Electrospray ionization (ESI) as a technique to transfer large glycoproteins in intact form from the liquid phase into the gas phase at atmospheric pressure, II. Two SIMS (secondary ion MS)-based techniques: vacuum matrix-assisted laser desorption ionization (vMALDI) and desorption ESI (DESI) as techniques to transfer large underivatized carbohydrates as well as glycoproteins from the solid phase into the gas phase at different pressure regimes, III. Analyzer for separating ionized sugar-containing species in the gas-phase as linear time-of-flight with ultra high mass detector, reflectron, ion mobility and quadrupole-reflector, IV. In the last part of the lecture a few successful examples will be presented as well as some potential pitfalls.

139: Mass spectrometric analysis of methylated monosaccharides and oligosaccharides

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Despite recent improvements in mass spectrometric analysis of carbohydrates, saccharide linkage analysis using permethylation of glycans in conjunction with combined gas chromatography/mass spectrometry is still a valuable tool in the field of carbohydrate structure analysis. To this end, permethylated oligosaccharides are cleaved by acid hydrolysis and the resulting monosaccharide derivatives are reduced and peracetylated. The partially methylated alditol acetates thus obtained are separated by gas-liquid chromatography and identified on the basis of their characteristic mass spectra generated by electron impact fragmentation. In addition, permethylated sugar chains may be directly analyzed by tandem mass spectrometry (MS/MS) or multiple cycles of ion isolation and fragmentation (MS^n), also providing important structural information. The great advantage of the latter approach is that glycan mixtures need not to be fractionated by laborious and time-consuming chromatographic procedures prior to analysis, but can be directly analyzed. In this lecture, a few examples will be discussed outlining the great power of both techniques.

140: To label or not to label - Is that the question?

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Analysis of fluorescently labeled oligosaccharides by hydrophilic-liquid interaction chromatogaphy (HILIC) has become a standard tool in glycobiological research and biotechnological quality control. Peak identification from unknown samples still requires reference glycans, glycosidase digests and off-line MS or a pinch of blind trust. Therefore, there is interest in LC-MS combinations using either labeled or underivatized oligosaccharides. This paper values the benefits of 15 different reagents for reductive amination with regard to fluorescence, ESI- and MALDI-MS and of different procedures for reagent removal. The behaviour of these derivatives on HILIC, RP and PGC columns was tested. Only a few of the tested derivatives, especially 2-aminopyridine, appeared as useful for RP- and PGC-LC and hence for refined fractionation and analysis.

An interesting alternative is offered in the form of analysis of non-labeled glycans by LC-ESI-MS on graphitic carbon, a technique equally suitable for N- and O-linked glycans. Changes in the relative abundance of sialic acid linkage isomers are easily detected using PGC-LC-MS as demonstrated with human chondrocytes subjected to pro-inflammatory cytokines.

Examples will be given of how PGC-LC-MS can yield information on the true structure using retention times of reference compounds and MSMS spectral matching. The advantages and limitations of quantitative analysis based on PGC-LC-MS will be discussed. The talk shall facilitate a decision for labeled- or unlabeled glycan analysis for particular tasks.

141: Selective enrichment of sialylated glycopeptides from biological samples for quantitative glyco-proteomics.

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Sialic acids constitutes the terminal monosaccharide of cell surface glycoconjugates and aberrant sialylation is involved in several diseases and in cellular development. Several methodological approaches in sample preparation and subsequent analysis using mass spectrometry have enabled the identification of the glycosylation sites and characterization of glycan structures. Here we describe a protocol for the characterization of sialic acid-containing glycopeptides using the high selectivity of titanium dioxide (TiO_2) towards sialic acid containing glycopeptides in combination with hydrophilic interaction chromatography (HILIC) and high performance mass spectrometry (MS). The selectivity of TiO_2 towards sialic acid containing glycopeptides is achieved in low pH buffers including a

substituted acid such as glycolic acid in order to exclude acidic peptides from the TiO_2 resin. By combining TiO_2 enrichment of sialylated glycopeptides with HILIC separation of the deglycosylated peptides, a more comprehensive analysis of formerly sialylated glycopeptides by MS can be achieved. We illustrate the applicability of this method to various biological materials in order to characterize glycoproteins that change in abundance or glycosylation during cellular transformation or development and further show this methods utility for biomarker discovery in various body fluids. In addition, we have used the selectivity of TiO_2 towards sialylated glycopeptides to set up a method capable of characterize intact sialylated glycopeptides by MS in order to identify the glycan diversity on individual amino acids in glycosylated proteins.

142: Glycoproteins: MS analysis of O-linked glycans

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O-linked glycosylation, usually also referred to as mucin type glycosylation, is one of the main post translational modifications on secreted and membrane associated proteins. The heterogeneity of O-linked oligosaccharides makes the analysis of these types of molecules challenging, with various core types as well as terminal structures containing blood group antigens and Lewis type modification. With isomeric structures as well modifications with sialic acid and sulphate, it has been shown that graphitized carbon LC-MS offers a versatile approach for screening the both acidic as well as neutral O-linked oligosaccharides. This approach allows identifying differences between tissues, between proteins and identification of diseased induced oligosaccharide changes. The approach has also shown provide an efficient method to monitor the glycoquality of recombinant glycoproteins. LC-MS/MS in negative ion mode has shown to produce informative spectra allowing tentative structures to be assigned and giving insight into regulation of various glycosyltransferases between individuals as well as glycosyltransferases induced as response to inflammation. This workshop presentation will provide information of how to prepare samples all the way from tissue to subjecting the O-linked oligosaccharides to mass spectrometry. Examples will be provided of how to interpret mass spectra from mucins isolated from natural sources, and from recombinant glycoproteins expressed in various expression systems. The use of on-line resources to assign compositions from full scan MS profiles will be demonstrated and the interpretation the data obtained from the fragmentation will elucidated.

143: Glycoproteins: MS analysis of N-linked glycans

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Recent developments in mass spectrometric analysis of N-glycans are presented: 96-well plate sample preparation protocols employing labeling by reductive amination are now available for high-throughput N-glycan analysis. Miniaturization is achieved using micro-SPE methods and nano-separation techniques. HILIC-UPLC of N-linked oligosaccharides featuring vastly increased separation power has recently been introduced and may be combined with fluorescence detection and mass spectrometry. Structural analysis of N-glycans by tandem mass spectrometry as sodium adducts or in negative-ion mode (deprotonated form or anion adduct) is greatly facilitated by software tools such as GlycoWorkbench which are freely available to the scientific community. These recent developments have boosted the role of mass spectrometry for N-glycosylation analysis in biology, medicine, and biotechnology.

Euroglycoscience Forum Crystallography & Modelling Workshop (Wednesday)

144: Leveraging Glycan Array Data with Computational Carbohydrate Grafting to Define the 3D Structure of an Anti-Tumor Antibody in Complex with Carbohydrate Antigen

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Conceptual novelty and the enabling potential of the technology Ideally, experimental methods such as X-ray crystallography and NMR spectroscopy are employed for understanding the binding specificity of a protein-ligand complex, however, they face enormous challenges when applied to the characterization of oligosaccharide-protein complexes. Biologically relevant glycans are often refractory to crystallization and difficult to obtain in large quantities. Conversely, theoretical methods are capable of generating 3D structures of protein-ligand complexes, and there is considerable interest in employing virtual ligand docking studies to predict the structures of carbohydrate-antibody complexes, due to the diagnostic and therapeutic relevance of antibodies. However, such predictions are difficult to validate, due to a paucity of experimental constraints. Recently, a new source for carbohydrate specificity data has emerged through the extensive development of glycan microarrays. Glycan array screening provides rapid insight into binding specificities, limited only by the number of elements in the array, the largest of which currently contain on the order of 500 members. Although such data provide no direct insight into the

nature of the 3D structure of the complex responsible for the observed specificities, they may be employed as constraints against which to validate theoretical 3D models for the complexes. Here we employ a new technology, Computational Carbohydrate Grafting (CCG), to generate 3D models of glycan-antibody complexes that are consistent with the data from glycan array screening. Given any structure for the protein in complex with a carbohydrate ligand, CCG can be employed to generate a 3D model of an intact glycan in the complex by splicing the additional branches of the glycan into the bound fragment. Here we demonstrate that the initial structure for the carbohydrate-protein complex can itself be generated using virtual ligand docking. The theoretical poses of bound glycans are then validated by comparison with specificities predicted by experimental glycan array data. This virtual approach overcomes the experimental challenges of generating structures for antibody-carbohydrate complexes. In addition, by grafting novel glycan structures through a validated model for a known binding epitope it is possible to expand the CCG approach to glycans that are not currently present on experimental microarrays, and to predict previously unexpected ligand specificities.

145: The Molecular Modelling Facets of Glycoscience

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Determination of the 3D structure of complex carbohydrates and understanding the molecular basis of their associations and interactions represent the main challenges of structural glycosciences. Elucidation of the 3D structures and the dynamical properties of oligosaccharides is a prerequisite for understanding the biochemistry of recognition processes and for the rational design of carbohydrate-derived drugs. The elucidation of their different structural levels are required to relate structure to properties. Some polysaccharides are also carriers of biological information that can only be deciphered if their interactions with other biological macromolecules are understood. Oligosaccharides, either in their free form or as part of glycoconjugates, are difficult to crystallize; structural data from X-ray studies are sparse. In solution, the flexibility of certain glycosidic linkages produces multiple conformations co-existing in equilibrium. The use of several spectroscopic methods is necessary for analysis of the conformational behaviour of such molecules. Polysaccharides differ from other biological macromolecules as the diffraction data that can be obtained are not sufficient to permit crystal structure determination. Procedures for molecular modelling of carbohydrates and carbohydrate polymers have been devised as an important tool for structural studies of these compounds. Various molecular modelling methods have been developed and have been widely used for the determination of oligosaccharide and polysaccharide conformations. The progress now allows for the simulation of carbohydrates in their natural environment, i.e. solvated in water or in organic solvent, in concentrated solution or in the binding site of a protein receptor. The different concepts and tools underlying the assessment of the structural and dynamics features of complex carbohydrates throughout molecular modelling procedures will be presented, taking into consideration their relevance to significant biological functions.

146: Computational modeling on the catalytic cycle of inverting β-1,4-galactosyltransferase-1

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The enzyme β-1,4-galactosyltransferase-1 (β4Gal-T1) catalyses the transfer of a galactose residue from UDP-Gal to the C4-hydroxyl group of N-acetylglucosamine. The catalytic activity of this enzyme was completely abolished when the second aspartic acid Asp252 of the DXD motif was replaced by asparagine (D252N mutant). This implies that Asp252 which does not directly interact with the Mn^{2+} ion co-factor in the active site of the inverting β4Gal-T1, plays an important role in the catalytic cycle of the enzyme. To understand function of Asp252, pK_a calculations at the empirical level were performed to predict protonation states of acidic amino acids in the empty active site of β4Gal-T1 as well as with the Mn^{2+} ion, donor and acceptor substrates bound. The proposed arrangement was tested by modeling the enzymatic reaction step of a galactose residue transfer from the UDP-Gal donor to the N-acetylglucosamine acceptor with density functional theory (DFT) methods at the hybrid QM/MM level. The results show that the pK_a value of Asp252 alters depending on the presence (or absence) of substrate molecules in the active site. In apoenzyme and with the Mn^{2+} ion co-factor bound, Asp252 prefers ionized aspartate form, while upon binding the donor and acceptor substrates it prefers protonated aspartic acid form. The subsequent QM/MM calculations indicated that Asp252 is not functioning as the catalytic acid in the chemical step of the catalytic cycle of β4Gal-T1. Consequently, the observed loss of the catalytic activity of the D252N mutant can be attributed to an inhibition of the catalytic process by releasing/binding the substrates from/to the active site rather than by the inhibition of the chemical step of the glycosidic bond transfer. Financial support for this research was granted by the Scientific Grant Agency of the Ministry of Education of Slovak Republic and Slovak Academy of Sciences (projects VEGA-02/0176/09 and VEGA-02/0101/11) and the Slovak Research and Development Agency (projects APVV-0117-06, APVV-0366-07).

147: Advices to determine and refine X-ray structures of protein-glycan complexes and glycoproteins

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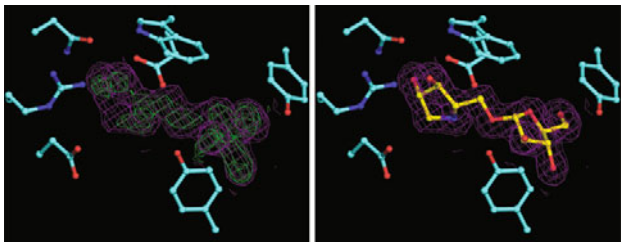
The strategies to solve and refine X-ray structures of glycoproteins or protein-glycan complexes are described.

Methods to obtain crystals of protein-glycan complexes are presented as well as the use of selenium glycan derivatives to solve the phase problem in carbohydrate binding proteins. Prior knowledge on the glycan structure is required for its proper refinement and modeling in the electron density: monosaccharide composition, hetero-compound 3-letters code, links, modified amino acid. It is indeed essential to use appropriate and correct sugar coordinates and libraries. Details are given on coordinates databases, ligand checking programs and how sugars libraries are working. It is important not to misinterpret or overinterpret the ligand electron density.



148: Computational studies of carbohydrate-antibody interactions using docking, site-mapping and conformational filters

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Antibodies that bind carbohydrates play central roles in immunity to pathogens, transfusion and transplantation rejection and are of emerging importance in the immunotherapy of cancer. While X-ray crystallography and NMR can provide detailed three-dimensional (3D) structural data on carbohydrate-antibody interactions, complementary computational approaches are often required. We have developed computational strategies based around the population-based analysis of a series of poses obtained from molecular docking. Initially, an ensemble of docked carbohydrate poses is generated in the 3D structure of an antibody binding site. The docking step was optimized and validated against a diverse set of crystal structures of carbohydrate-antibody complexes [J Chem Inf Model (2009) 49:2749-60]. A "site-map" that takes into account all docked poses is used to identify antibody residues that are most likely involved in carbohydrate recognition [Mol Immunol (2009) 47:233-246]. Epitope maps reveal the carbohydrate atoms frequently participating in interactions with antibody. Conformational analysis of glycosidic linkages suggests families of favored antibody-bound carbohydrates. By combining the output of these three approaches, we can systematically identify of the most likely carbohydrate binding modes using purely in silico techniques [Glycobiology (2010) 20:724-35]. We illustrate the computational approach with carbohydrate recognition by a panel of xenoreactive antibodies. Site maps show carbohydrate recognition occurs in structurally conserved regions of the binding sites. Preferred binding modes suggest the importance of the terminal disaccharide epitope as a binding anchor, and were also able to explain the selectivity of certain antibodies for Glc over GlcNAc in the third position of the carbohydrate chain. This work was supported by funding from the National Health and Medical Research Council of Australia (NHMRC).

149: A Weak Affinity Dynamic Microarray for Glycan Profiling: modeling and preliminary experimentation of a high-throughput tool for screening and profiling glycoproteins

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Lectin microarrays have been proposed as a platform for profiling glycoproteins, where each lectin has a few, highly-specific glycan pairs. It may be possible to profile using a much smaller set of lectins, if the entire affinity spectrum can be utilized. A Weak Affinity Dynamic Microarray (WADM) utilizes transducers that allow for single molecule adsorption and desorption dynamics to be measured in real time, as opposed to equilibrium binding only. This resolves both weak and strong-binding partners. To illustrate, we use approximate binding constants from 75 lectins and 442 glycans to define the requirements and limitations of the dynamic array concept. A Kinetic Monte Carlo model of reduced glycoproteins can estimate the optimal number of lectin types, number of transducers, and glycoprotein concentration for three prototypical glycan profiling applications: 1) screening protein therapeutics, 2) clinical biomarker screen, and 3) complete profiling of glycoproteins without a priori knowledge of their synthesis pathway. Experimental considerations for profiling homogenous and heterogeneous solutions of glycoproteins with a lectin WADM are discussed as well as using the dynamic array to obtain an accurate database of mono and multivalent interaction parameters between lectin-glycan pairs. Finally, we present our current experimental work on building a WADM using a single-walled carbon nanotube (SWNT) based optical sensor. The SWNT are solubilized in a dilute solution of chitosan and linked to the lectin sensor protein by a novel chemical tether. The proximity of this tether to the SWNT quenches the fluorescence of the excited nanotube. We observe quenching responses to analyte glycoprotein probes, biotinylated glycans bound to streptavidin. The preliminary interactions studied in this work are between the GafD lectin and GlcNAc glycan as well as GBP1 lectin and Galb glycan at concentrations ranging from 10μM to 10nM. We discuss the current difficulties of differentiating like-glycan signals and new experimental techniques to overcome these challenges.

150: Establishment of the principles of flax rhamnogalacturonan I three-dimensional organization by computer modeling

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The terms secondary and tertiary structure in respect of polysaccharides is not widely applied because of its irregular molecule construction. Nevertheless, there is evidence for the existence of the high level spatial organization of polysaccharides. In particular, the ability of phloem flax fiber galactan to maintain the hydrodynamic volume after a substantial decrease of molecular weight was found. This polysaccharide is built as a complex rhamnogalacturonan I with long side galactose chains. We assume that the three-dimensional galactan organization is based on the ability of its molecules to form associates. We have tried to establish by variety of approaches the structure of the minimal galactan fragment able to association. According to NMR spectroscopy data, the polysaccharide backbone has greater mobility compared to the side chains. This suggests that the polysaccharide backbone is located on the agglomerate periphery, and the interacting side chains are directed into the core-zone. The minimal fragment of the associate backbone containing six rhamnose residues was indicated by hydrolysis with rhamnogalacturonanhydrolase. The following basic characteristics of the minimal galactan fragment necessary for self-assembly was indicated by computer modeling (ChemOffice 2005, HyperChem 8.07): a) the length of the fragment side chain should be at least five galactose residues, which determines its structure as a single loop of right-handed helix that can interact with other galactose chain, b) galactose chain located at one side relative to the backbone, c) organization of the polysaccharide molecules eliminates the connection of long side chains to the two neighbouring rhamnose residues, because in this case, the chain due to their repulsion would be aimed in different directions and the backbone would not have greater mobility compared to the galactose chains. The established characteristics may be universal for a number of polysaccharides; further study of the minimal fragment construction will help to come closer to understanding the principles of three-dimensional organization of polysaccharide molecules.

Euroglycoscience Forum Prokaryotic Protein Glycosylation Workshop (Wednesday)

151: *Neisseria* species as a model system for bacterial O-linked protein glycosylation

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Although protein glycosylation systems are becoming widely recognized in bacteria, little is known about the mechanisms and evolutionary forces shaping glycan composition. Species within the genus Neisseria display remarkable glycoform variability associated with their O-linked protein glycosylation (*pgl*) systems and provide a well-developed model system to study these phenomena. We found that in addition to *N. gonorrhoeae* strain N400[1], other gonococcal strains as well as isolates of *N. meningitidis* and *N. lactamica* express broad spectrum O-linked protein glycosylation[2]. All glycoproteins identified in *N. gonorrhoeae* strain N400 are predicted to be lipoproteins or integral membrane proteins localized to the periplasm or cell surface[2]. Recently, we focused on answering two outstanding questions in this system: 1) What is the overall level of glycan diversity in *Neisseria* species? and 2) What is the genetic bass underlying the glycan diversity observed? Accordingly, we have developed an approach employing genetically defined recombinant backgrounds, mass spectrometric analyses and glycan serotyping and used these methodologies to examine a large set of isolates of *N. gonorrhoeae*, *N. meningitidis* and *N. lactamica*[2]. The results indicate that the levels of pan glycan diversity are greater than previously appreciated with at least 13 different protein - associated glycoforms being identified amongst the species. Within a single strain, up to seven glycoforms can be expressed. In addition, some *pgl* genes are highly polymorphic and encode products with varied levels of activity. These findings have allowed us to document a novel role for intrinsic genetic interactions in shaping glycan evolution in a protein glycosylation systems.

References
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2. B. Børud et al., J Bacteriol, 2010, 192(11):2816-2829.

152: Elucidation of the N-glycosylation pathway in the crenarchaea

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Historically it was long been believed that glycosylation is a phenomenon restricted to Eukarya, however, when in 1976 Mescher and Strominger purified the S-Layer protein from *Halobacterium salinarum* which contained glycans covalently linked to asparagine residues, questions evoked how N-glycosylation occurs in Bacteria and Archaea. Today N-glycosylation is thought to be conserved across all three major domains of life. During the last years substantial progress in describing N-glycosylation pathways in three euryarchaeota, one major archaeal

kingdom, has been made. Although eukarya, bacteria, and archaea all seem to have certain characteristics of the N-glycosylation pathway in common, archaea display a mosaic of features from the eukaryal and bacterial system. However, so far the N-glycosylation process in crenarchaeota is still uncovered. Here we will report the first results elucidating the N-glycosylation pathway in the thermoacidophilic archaeon *Sulfolobus acidocaldarius*. The N-glycosylation pathway in *S. acidocaldarius* shows some significant differences compared to these of the other archaea, e. g. scattered gene localization of glycosyltransferases (GT), which imposes a challenge to identify GTs involved in glycosylation processes. In contrast to the non essential N-glycosylation pathway in the studied euryarchaeota, the N-glycosylation pathway is essential for the survival of *S. acidocaldarius*. Further *S. acidocaldarius* exhibited a unique composition and branched structure of the N-linked oligosaccharide, so far not found in other archaea.

153: Prokaryotic Glycoprotein Database (PROGPDB): A database of experimentally characterized glycoproteins of prokaryotic origin.

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Glycosylation is a recently identified important post translational modification of proteins in prokaryotes. Different studies have implied different roles for prokaryotic glycoproteins in cellular/ extra cellular milieu including a direct correlation with the virulence of the pathogenic bacterial species like *Camplyobacter*, *Mycobacteria* etc. Consequently the research interest has now increased exponentially towards both basic and applied aspects of the glycosylation of proteins in archaea and eubacteria. Prokaryotic Glycoprotein Database (PRO GPDB) therefore has been developed with an aim to aid/further such studies by providing a comprehensive, extensively manually curated repository of experimentally characterized prokaryotic glycoproteins in a highly cross-referenced, largely non-redundant format. PRO GPDB is the first open access database dedicated exclusively to eubacterial and archaeal glycoproteins. The databse is presented as a searchable catalogue of experimentally characterized prokaryotic glycoproteins defined as entries with known glycosylated residues. PRO GPDB also lists the uncharacterized prokaryotic glycoproteins defined as entries with known glycosylation but unknown glycosylated residues. Currently more than 260 experimentally validated entries comprising both characterized as well as uncharacterized prokaryotic glycoproteins are compiled in the database. Corresponding 243 literature references are arranged in a logical manner in the database summarizing the best of the published reviews on a separate page. Further each glycoprotein entry contains information in single page user friendly manner with text/ embedded hyperlinks/ pictures detailing information on taxonomy, gene, protein, Protein function/ structure, attached glycan/ structure, associated (predicted/ characterized) OST/ other glycosyltransferases along with the experimental information pertaining to the characterization of the glycosylation site and supporting references. Discrepancies in the positions of the glycosylated residues in full length proteins are appropriately addressed according to the published experimental literature. The database can be browsed by multiple parameters and supports a user-friendly text search tool, database blast tool and links for relevant existing databases as well as available tools to study glycoproteins.

154: Glycoproteomic analysis of O-linked glycans from the oral pathogen, *Tannerella forsythia*

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Tannerella forsythensis is an anaerobic Gram-negative pathogen of the *Cytophaga-Bacteroides-Firmicutes* cluster of bacteria. It is implicated in periodontal diseases like periodontitis. Two glycoproteins that build up a two dimensional crystalline surface layer (S-layer) were identified as major virulence factors of *T. forsythensis* [1]. Here we present details on the glycosylation of these two S-layer proteins. The analytical work was done by ESI-MSMS coupled to micro-flow liquid chromatography using PGC- and RP-columns [2] in combination with 600 MHz ^{1}H NMR spectrometry. O-glycosylation was shown to occur at an amino acid motif that was recently found in other Bacteroides [3]. Further the same type of glycosylation was not only found on S-layer proteins, but also on many other *T. forsythia* proteins. The S-layer oligosaccharides consist of eight sugar residues, including, among others, hexuronic acids, deoxy/dideoxy-hexoses, fucose, and a sialic acid-like derivative. Thus, Tannerella forsythia features bacterial protein O-glycosylation encompassing so far unique structural elements [4]. The work is supported by the Austrian Science Fund, project P21954-B20 (CS).

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155: The outer membrane vesicle glycoproteome of the periodontopathogen *Tannerella forsythia*

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Tannerella forsythia (*Tf*) is an anaerobic, Gram-negative pathogen which was originally isolated from the human oral cavity of periodontal disease patients. As a member of the "red complex" bacteria, it is implicated in the pathogenesis of periodontitis and may also exert a long-term impact on systemic disorders such as cardiovascular disease [1]. Even though the mechanism of *Tf* infection has yet to be elucidated, putative virulence factors are beginning to be adequately identified and characterized. In the light of these findings, outer membrane vesicles (OMVs), ubiquitously shed from Gram-negative bacteria, have come under increased scrutiny. OMVs have been shown to contribute to the pathogenicity of many bacteria by enriching virulence factors and delivering them over long distances, superseding direct bacterial contact with their host [2]. It is therefore conceivable that *Tf* OMVs play a role in the shuttling of virulence factors and affect the development and progression of periodontal disease.

Here, we present the status of research concerning the following aspects: (i) isolation of *Tannerella forsythia* OMVs and first TEM images, (ii) the composition of *Tf* OMVs, and (iii) a preliminary analysis of their glycosylated cargo.

This information represents the initial steps towards characterizing of *Tf* OMVs at the molecular level. In conjunction with future experiments, our findings may help to elucidate the outer membrane vesicles' role in pathogenesis, their impact on the physiology of *Tannerella forsythia*, and their influence on the organism's lifestyle. This will eventually pinpoint novel targets for the treatment of periodontal disease.

The work is supported by the Austria Science Fund, projects P20605-B12 and P21954-B20 (to CS) and BioToP W1244 to (PM & CS).

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Euroglycoscience Forum Glycolandscape Engineering Workshop (Wednesday)

156: Towards cell glycolandscape

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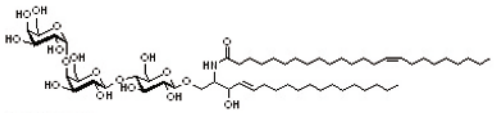
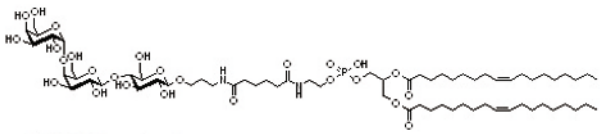
In order to effectively study the influence that specific glycolipids have at the cell surface ideally the glycan of interest present on that cell would have a defined structure, preferably be chemically engineered, and be at a specific and known density. By nature of their lipid tail glycolipids have a natural ability to self-insert in cell membranes, but often the requirement to use solvents to maintain them in a solution phase suitable for insertion can limit their compatibility with live cells, particularly fragile cells such as embryos. Additional important restriction is very hard and multistep chemical synthesis of completely natural glycosphingolipids bearing ceramide lipid moiety. Recently synthetic glycolipid-like constructs, which have a spacer between more simple and synthetically friendly lipid and glycan head have been constructed. Synthetic glycolipids dispersible in biological media are capable of incorporating into cell membranes in vitro and in vivo, and thus have the ability to create novel artificial glyco-landscapes, or specifically point-wise modify natural glyco-landscape on living cells. Using a variety of different glycans ranging from disaccharides to polysaccharides together with different length and high hydrophilicity spacers, we designed a series of synthetic constructs. Contacting these constructs with cells created cells with controlled glycan density and/or altered biological function.


natural Gb3


synthetic Gb3 construct

157: Switchable glycomimetics: Conformational control of bacterial adhesion

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Glycoconjugates on cell surfaces play an essential role in a variety of biochemical communication events. The molecular mechanisms forming the basis of these processes are not yet fully understood. In this regard, structure-function relationships are investigated in glycobiology to shed light on the biological role of cell surface carbohydrates. Recently, attention has been drawn to the field of conformational control within the macromolecular array of the cell surface glycoconjugates. Structural changes occurring on cell surfaces, can be induced by

carbohydrate-protein interactions, i. e. to launch specific biological processes. Modification of biologically relevant substances by introducing small molecular 'photoswitches' is a promising tool to advance the understanding regarding the role of conformational settings. Hence, to get an idea about the role of conformatonal control in carbohydrate recognition, it has become our aim to synthesize photoswitchable glycomimetics such as the glycothymidines or azobenzene glycosides, respectively. Both are sensitive to external stimuli, particularly light. This would eventually allow us to alter the conformational flexibility of a supramolecular glyo environment and investigate the impact of conformational changes on its biological functioning. Thymidine has been chosen as a photochemical switch allowing [2+2]-cycloaddition. The nucleoside serves as photosensitive biocompatible scaffold with orthogonal functionalities, to be modified with carbohydrate moieties and to be immobilized. Azobenzene, on the pother hand, allows photochemical E/Z isomerization and thus alteration of carbohydrate exposure on a scaffold or surface, respectively. To investigate potential changes in carbohydrate-protein interactions upon irradiation of the photosensitive glycoconjugate mimetics, mannose-specific bacterial adhesion has served as test system.

158: Target-selective photodegradation of oligosaccharides by designed small organic molecules

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Carbohydrates play crucial roles in a wide range of biological processes, including serious diseases, and have been considered the third class of information-encoding biological macromolecules. Therefore, glycomics—an analogue of genomics and proteomics—has attracted much attention in fields such as chemistry, biology and medicine. Despite its importance, however, progress in this field has not been particularly rapid, because there has been comparatively little research on the development of tools for selective control of specific functions of oligosaccharides. Here, we demonstrate innovative methods for target-selective photodegradation of oligosaccharides by light-activated small organic molecules (anthraquinone-boronic acid hybrids) without any additives under physiological conditions. To the best of our knowledge, this is the first example of target-selective degradation of oligosaccharides by designed small organic molecules using light switching under neutral conditions. This novel tool may serve as a starting point for bottom-up designs of photodegradation agents for specific oligosaccharides, which may facilitate further understanding of the structure-activity relationships of oligosaccharides and the development of novel therapeutic drugs targeting oligosaccharides.

159: Enhanced potencies of multivalent carbohydrates on microarrays and magnetic nanoparticles

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The display of carbohydrates on surfaces provides new tools for the study of protein carbohydrate interactions. In this context we worked on the display of glycostructures on a chip surface and on magnetic nanoparticles. We have attached glycodendrimers to a chip surface for the study of multivalency effects. Which is an important phenomenon in protein-carbohydrate interactions. Valencies were varied from 1 to 8 and corrections were made for the valencies. A series of fluorescent lectins was evaluated and for each of them a binding profile was obtained from a single experiment showing both the specificity of the lectin for a certain sugar and also whether or not it prefers multivalent ligands or not. Very distinct binding patterns were seen for the various lectins. The magnetic nanoparticles were used for the purpose of bacterial detection. The rapid identification and characterization of bacterial pathogens is an important goal to serve as an alternative to time-consuming culturing. Bacteria carry adhesion proteins on their surface and these are crucial to the infection process. The binding specificity of bacteria is a useful handle on the outside of the pathogen that can be taken advantage of in this context. Detection of the zoonotic bacteria pathogen Streptococcus suis was achieved using magnetic glycoparticles. New developments in both areas will be discussed.

160: Synthetic Glycocluster – Tuning Avidity and Selectivity by Topology

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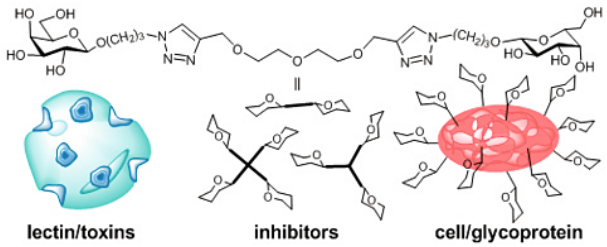
Tuning biological events via multivalent recognition processes is widely spread in Nature. Clustering carbohydrate-recognising structures, e.g. lectins, with multivalent carbohydrate ligands forming heterogeneous cross-linked materials can induce various biological events such as immune regulation, cell-growth regulation or cell-cell recognition.[1]

To get control over selectivity and avidity in carbohydrate recognition processes while using synthetic ligands advances not only the understanding of underlying principles but also increases the therapeutic and diagnostic perspective of such compounds.[2]

In order to investigate the impact of multivalency, the spacer system, and the scaffold, i.e. features that eventually can lead to rational inhibitor design, we prepared a series of 16 di- to tetra-valent glycoclusters

carrying either a common head group (Gal/Lac/LacNAc) or maltose as a negative reference. Click type chemistry followed by deacetylation provided the glycoclusters in moderate to excellent overall yields. The clusters were evaluated with a number of receptor systems, e.g. the plant toxin *Viscum album* L. agglutinin, Galectin-3, the truncated form of Galectin-3, and Galectin-4.[3]



Comparing the relative inhibition capacities of subsets of glycoclusters carrying the same sugar head group revealed distinct inhibition profiles. During the presentation both synthetic aspects and biological results will be discussed.

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161: Cholesterol masking of plasma membrane glycosphingolipids

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Glycosphingolipids(GSLs) are key membrane receptors involved in cellular interactions, differentiation and microbial pathogenesis. In model and cell membranes GSL can accumulate in cholesterol enriched 'lipid rafts'. We have found that in cell derived and model membranes, the interaction between GSLs and cholesterol can prevent the binding of appropriate ligands e.g. anti-GSL antibodies, toxins. Molecular modeling studies have shown that this GSL-cholesterol interaction can alter the conformation of the carbohydrate, such that the sugar is presented in a membrane parallel, rather than perpendicular orientation. The membrane parallel format could restrict ligand access. Cholesterol extraction from human tissue sections indicate that cholesterol masking of GSL antigens may be a common feature of GSL membrane presentation in vivo. Since tumour cells have higher levels of cholesterol than normal cells, we have begun to study whether cholesterol masking of plasma GSL antigens could be a means by which tumours evade immune surveillance. Our studies indicate that this may be the case. GSL markers of stem cells can be detected after cholesterol extraction of human tumour biopsies. Significantly globotriaosyl ceramide(Gb3), the GSL receptor for verotoxin (Shiga toxin) which we and others, have reported to be upregulated in many human cancers, and proposed verotoxin as an antineoplastic, can be unmasked in some previously immunohistologically Gb3 negative tumours. The receptor function of GSLs is dependent not only on the structure of the carbohydrate but the ceramide moiety also. The modulation of GSLs by cholesterol may prove the most significant example of this "aglycone effect".

Poster Session I (Monday)

Carbohydrates & Disease

162: Elevated O-GlcNAc modification of proteins in diabetic kidney

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Increased flux through the hexosamine biosynthesis pathway promotes the modification of proteins by O-linked N-acetylglucosamine (O-GlcNAc). In our previous immunohistochemical study, we demonstrated that the extent of O-GlcNAcylation was increased in kidneys from diabetic GK rats. In the present study, to identity marker proteins that change in their extent of O-GlcNAcylation in the diabetic kidney from GK rats, we separated total kidney proteins by two-dimensional gel electrophoresis. O-GlcNAcylated proteins that changed significantly in the degree of O-GlcNAcylation were identified as cytoskeletal proteins (alpha-actin, alpha-tubulin, alpha-actinin 4, myosin) and mitochondrial proteins (ATP synthase beta, pyruvate carboxylase). This is the first report to show that alpha-actinin 4, whose mutation causes familiar segmental glomerulosclerosis (FSGS), is O-GlcNAcylated. Results of immunoprecipitation and immunoblot studies, as well as in situ Proximity Ligation Assays demonstrated that the extent of O-GlcNAcylation of the above proteins increased in the diabetic kidney. Immunofluorescence studies revealed that the levels of immunoreactivity of actin, alpha-actinin 4 and myosin were markedly increased in the glomerulus of the diabetic kidney. In contrast, the level of immunoreactive alpha-tubulin was concomitantly decreased in the proximal tubule of the diabetic kidney. Immunoelectron microscopy revealed that immunolabeling of alpha-actinin 4 increased in the foot process of podocytes. These results suggest that changes in the O-GlcNAcylation of cytoskeletal proteins are closely associated with the morphological changes in the podocyte

foot processes in the glomerulus and in microvilli of proximal tubules in the diabetic kidney.

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163: Crystallization and preliminary X-ray diffraction analysis of alpha mannosidase LM408 cloned from *D. melanogaster*

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Class II α-mannosidases exhibit exo-type activity toward α-1,2-, α-1,3- and α-1,6-mannose linkages and based on sequence similarity are classified into glycoside hydrolase family 38. Several eukaryotic class II α-mannosidases have been expressed and characterized and lysosomal and cytosolic α-mannosidases play an important role in the degradation of the asparagine-linked carbohydrates of glycoproteins. Deficiency of lysosomal mannosidase in humans leads to the lysosomal storage disorder – α-mannosidosis. To date, there is no available crystal structure for insect lysosomal α-mannosidase although several mannosidases from this family have been crystallized. Recombinant α-mannosidase LM408 of *Drosophila melanogaster* was cloned based on the peptide similarity with human and bovine lysosomal mannosidase. The enzyme activity was proven using synthetic pNP-α-mannopyranoside and substrate specificity was determined using oligomannose Man 8 and Man 9. Prior to crystallographic study, the N-terminal poly-histidine tagged form of LM408 was heterologously expressed in Pichia pastoris. The recombinant enzyme was observed to be post-translationally processed into multiple peptide forms. SDS-PAGE analysis and deglycosylation studies confirmed occupation of the N-linked glycosylation sites and possible dimerization of the molecule. The crystallization conditions were initially screened by means of the counter-diffusion method using the sparse-matrix kit GCB-DOMINÓ. Very faint plate-like crystals were obtained after several days from PEG and sodium cacodylate as precipitating agents. Crystals were flash-cooled and tested for diffraction at the SSRL. The observed resolution was worse than 4 Å as crystals of such large assemblies often diffract weakly. After preliminary X-ray analysis the orthorhombic space group P222 was found. This work was supported by the Slovak Research and Development Agency APVV (0117-06), the Ministry of Education of the Slovak Republic and Slovak Academy of Sciences - VEGA (2/0095/08) and the Austrian Fonds zur Förderung der wissenschaftlichen Forschung (L314).

164: Prognostic role of heparan sulphate 3-O sulphotransferase 3A1 (HS3ST3A1) in breast cancer: an immunohistochemistry approach

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Heparan sulphate sulphotransferases are a family of enzymes involved in the synthesis of heparan sulphate. These enzymes transfer sulphate groups from the donor, phosphoadenosine phosphosulphate to various carbon positions on heparan sulphate. Aberrant regulation of sulphation will disrupt the synthesis of heparan sulphate and reports have shown that a deviant expression of sulphotransferases promotes progression of cancer. Using immunohistochemistry, we evaluated the associations between heparan sulphate 3-O sulphotransferase 3A1 (HS3ST3A1) and invasive ductal carcinoma (IDC) of the breast. Two hundred and fifty five IDC cases were assessed immunohistochemically and the results were correlated with clinicopathological and survival data. Size of tumour showed inverse association with intensity of expression of HS3ST3A1, where larger tumours showed under immunoexpression of HS3ST3A1. Staining pattern revealed weak HS3ST3A1 antibody staining intensity in high grade tumour whilst in low grade tumour, stronger immunoexpression was observed. Cerb2 negative tumours showed elevated expression of HS3ST3A1. Breast cancer without oestrogen receptor expressed higher amounts of HS3ST3A1. In terms of survival, oestrogen receptor negative IDC cases with higher immunoexpression of HS3ST3A1 have better disease free survival and overall survival compared to patients with lower immunoexpression of HS3ST3A1. In summary, HS3ST3A1 may serve as a prognostic indicator for IDC.

165: Prognostic value of heparan sulfate expression in gastric carcinoma

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Heparan sulfate is a glycosaminoglycan which consists of repeating disaccharide units of glucuronic acid/iduronic acid and N-acetylglucosamine. It is covalently attached to a core protein to form heparan sulfate proteoglycan. Heparan sulfate contains various sulfation patterns which determine its biological function. It acts as a regulatory cofactor for a variety of signaling molecules which is implicated in cancer. To determine if heparan sulfate has a prognostic value in gastric cancer, immunohistochemistry was used to examine its expression patterns in 162 cases of gastric carcinoma specimens in tissue microarray format by using the 10E4 antiheparan sulfate monoclonal antibody. The expression of heparan sulfate in both epithelial and stromal components were examined and correlated with clinicopathological parameters, including histological grade, extent of cancer infiltration, lymph node metastases, perineural invasion, lymphovascular invasion, perforation of gastric wall and stromal reaction. The expression of heparan sulfate was also analysed for significant association with patient survival. In our study, a lower expression of heparan sulfate in the epithelial component was found to be associated with higher histological grade and greater extent of cancer infiltration as well as decreased patient survival after tumour recurrence. These data suggest that heparan sulfate expression may play an important role in regulating gastric cancer phenotype and their expression may be of prognostic utility in gastric cancer.

166: Hybrid gangliosides for the detection of anti-ganglioside complex antibodies

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Gangliosides are glycosphingolipids that are concentrated in the outer leaflet of neuronal membranes with exposure of their oligosaccharides on the cell surface. Guillain-Barré syndrome (GBS) is acute autoimmune neuropathy, often subsequent to an infection. Antiganglioside antibodies are often closely associated with clinical phenotype and specific symptoms of GBS. Recent studies demonstrated that some GBS patients had serum antibodies that specifically recognize the novel glycoepitopes formed by two individual ganglioside molecules and named such antibodies as 'anti-ganglioside complex (GSC) antibodies'. Those antibodies can be used as diagnostic markers of GBS. Conventional measurement of antiganglioside antibodies has been done for purified single ganglioside antigens using enzyme-linked immunosorbent assays (ELISAs) or thin-layer chromatogram (TLC)-immunostaining. The availability of several hybrid gangliosides, containing two or more oligosaccharide chains and the availability of simple analytical approaches opens new perspectives for the understanding and therapy of several neuropathies. For this purpose we prepared the dimeric hybrid GM1-GD1a ganglioside derivative that contains two structural different oligosaccharide chains. A mixture of the two natural gangliosides was described to be recognized by the sera from patients with specific and clinical characterized GBS. We prepared the GM1-GM1 and GD1a-GD1a dimers to be used as controls. After removal of the acyls and reconstitution of the original acetylated sugars, the lyso-gangliosides, were connected with adipic acid to form the hybrid compound, a same-how mimic of the heterogeneous plasma membrane cluster of gangliosides. The dimeric hybrid GM1-GD1a was very well recognized by the GBS serum. However no reactivity was observed with the patient serum and the dimeric GM1 and dimeric GD1a. This suggests that both the GM1 and GD1a chain are necessary for a strong interaction and to maintain stable the antibody-antigen complex.

167: What is real target of galectin-3?

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Galectin-3 is a member of a family of β-galactoside-binding lectins which share significant sequence similarity in the carbohydrate recognition domain. To date, 15 members have been identified in mammals and named in the order of their identification. On the basis of structural architecture, they are classified into three types, namely, the prototype, chimera type, and tandem-repeat type. Galectin-3 is only chimera type galectin and that consists of one homologous carbohydrate recognition domain and connected by a linker peptide. They are involved in various biological processes, typically in immunological events. Most of the physiological activities of galectin-3 are believed to be triggered by the recognition of specific counterpart oligosaccharide ligands expressed on target cells, although it has been argued that galectin functions cannot simply be explained by such sugar-binding properties. During the course of our binding study by biosensing analysis, we found that human galectin-3 interacts with a particular bit of structure, even though, there are lacking beta-galactoside structure (lactose, and N-acetyllactosamine). Interestingly, the novel specific interaction strongly depended on the C-terminal carbohydrate recognition domain, because the addition of potent saccharide inhibitors abolished the interaction. Moreover, N-terminal

nonlectin domain also involved in the interaction, because the altered galectin-3 (only C-terminal carbohydrate recognition domain) showed weak interaction in compared with whole galectin-3. Biological and medical significance of the galectin-3 – glycoprotein (lacking beta galactoside structure) interaction observed will be discussed.

168: Clinical Evaluation of Sialyl-Tn Antigen Level on CA125 Core Protein in Patients with Endometriosis and Ovarian Cancer

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CA125 is commonly used as a diagnostic marker of ovarian cancer, but its elevated expression is also found in a number of benign conditions including endometriosis. Our aim was to develop an assay method for evaluating differentially glycosylated CA125 core protein (MUC16) in endometriosis and ovarian cancer patient, and in ovarian cancer of different clinical stages, cytological grades and histological types. In contrast to sialyl-Lea and Tn antigens, a negligible level of sialyl-Tn antigen was detected in CA125-enriched fractions obtained from peritoneal fluid of endometriosis patients. Thus, we developed a sandwich ELISA for determining the sialyl-Tn antigen level on CA125 core protein (sTn/CA125). The level of sTn/CA125 in ovarian cancer patients was significantly higher than that in endometriosis patients ($p < 0.001$), and was elevated in 44 % of ovarian cancer patents but not in all endometriosis patients. Furthermore, it increased more prominently in ovarian cancer patients than the level of CA125 as both the clinical stage and cytological grade advanced, and varied in histologically different ovarian cancer. Our present results indicated that estimation of the sTn/CA125 level could be useful for discriminating endometriosis from ovarian cancer, and for evaluating the clinical stage, cytological grade and histological type of ovarian cancer.

169: Expression of N-acetylglucosaminyltransferase IVa Regulates Invasion of Mouse Hepatocarcinoma Cells in vitro

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Branching structures in complex N-glycans are synthesized in the Golgi apparatus by N-acetylglucosaminyltransferase (GnT) -I to -VI on a common core structure of Manα1-6(Manα1-3)Manβ1-4 GlcNAcβ1-4GlcNAcβ1-Asn.
N-acetylglucosaminyltransferase (GnT)-IVa catalyzes the formation of the GlcNAcβ1-4 branch on the GlcNAcβ1-2Manα1-3 arm of the core structure of N-glycans. Therefore, GnT-IVa is one of the key glycosyltransferases regulating formation of tri- and other multiantennary structures. Previous studies showed that GnT-V, which catalyzes the formation of β1-6GlcNAc branching on N-glycans, is closely associated with malignant transfomations. While GnT-III prevents the metastatic spreading of cancer cells, which catalyzes the bisecting structure in N-glycans. Our recent studies indicated that the expression of GnT-IVa in Hca-F cells was much higher than that in Hepa1-6 cells, these two mouse hepatocarcinoma cell lines have high and no metastatic potential in the lymph nodes respectively. Then, the exogenous GnT-IVa was introduced in Hepa1-6 cells, meanwhile the expression of GnT-IVa was down-regulated in Hca-F by lentivirus induced RNAi. The results showed that the over-expression of GnT-IVa in Hepa1-6 cells changed the construction of N-glycans and furthermore increased the invasion capability in vitro. Conversely, downregulation of GnT-IVa in Hca-F cells significantly decreased the invasion capability in vitro. These results suggest that GnT-IVa could be acts as a key role in invasion and metastasis of hepatocarcinoma cells.
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170: TNFalpha, IL-6 and IL-8 control sialyl Lewis x and 6-sulfo sialyl Lewis x epitopes biosynthesis in human bronchial explants and in A549 lung cancer cells.

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Bronchial mucins from patients suffering from cystic fibrosis (CF) exhibit glycosylation alterations, especially increased amounts of the sialyl-Lewis^x (NeuAcα2-3Galβ1-4[Fucα1-3] GlcNAc-R, sLe^x) and 6-sulfo-sialyl-Lewis^x structures (1). These epitopes are preferential receptors for *Pseudomonas aeruginosa*, the bacteria responsible for the chronicity of airway infection of CF patients. Several studies have shown that inflammation may affect glycosylation and sulfation of glycoproteins, including mucins. We have shown that incubation of human bronchial mucosa with TNFα, IL-6 or IL-8 results in an increase in the expression of several α1,3/4-fucosyltransferases (FUT3, FUT11), α2,3-sialyltransferases (ST3GAL4, ST3GAL6), and GlcNAc-6-O-sulfotransferases

mRNA. The amounts of sLe^x and 6-sulfo-sLe^x epitopes at the periphery of glycoproteins were also increased (2). Identifying mechanisms by which (6-sulfo-)sLe^x structures are overexpressed on CF patients bronchial mucins may allow the identification of new targets to fight bronchial infection by *P. aeruginosa*. In order to study the transcriptional regulation of glycosyltransferases and sulfotransferases involved in sLe^x and 6-sulfo-sLe^x synthesis, we used A549 lung cancer cells as a cellular model: treatment of A549 cells with TNFa induced an increased expression of ST3GAL4, FUT3 and FUT11 mRNA, and increased amounts of sLe^x and 6-sulfo-sLe^x structures on glycoproteins, showing that A549 cells can be used as a cellular model for transcriptional regulation analysis. Type B1 and BX transcripts of the ST3GAL4 gene were the most expressed in A549 cells, and increased by TNFα treatment. Type BX transcript was also the major transcript in human bronchial explants and its expression increased by TNFα. We are now studying a potential promoter region governing the expression of BX/B1 transcripts, and the activation of different signaling pathways by TNFα in order to identify the mecanisms of TNFα-induced overexpression of the ST3GAL4 gene. This work is supported by the association "Vaincre la mucoviscidose" (IC0928). References (1) Groux-Degroote, S. et al., (2008) Biochem. J., 410, 213-223. (2) Lo-Guidice, J.M., et al., (1994) J. Biol. Chem. 269, 18794-18813.

171: Glycan biomarkers in gastric lesions: tissue and serum characterization

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Gastric cancer is the second leading cause of death by cancer worldwide [1]. Gastric cancer is, in general, preceded by a long carcinogenesis pathway which includes gastritis caused by *H. pylori* infection, chronic atrophic gastritis that may progress to intestinal metaplasia, dysplasia and ultimately gastric carcinoma. Several glycosylation alterations occur in these lesions of the gastric mucosa. The identification of glycan changes in the precursor lesions of gastric cancer is of high interest and could be used as a source for finding new biomarkers for early diagnosis applications [2]. This study describes the development of a glycoproteomics approach to identify glycoproteins with glycan alterations, expressed in gastric lesions, which could be used as novel risk markers for gastric cancer. The expression of two truncated glycans, T antigen (Galβ1-3GalNAcα1-O-Ser/Thr) and STn antigen (NeuAcα2-6GalNAcα1-O-Ser/Thr) in different gastric tissues (normal mucosa, gastritis, and intestinal metaplasia) was investigated and the expression of altered glycoproteins in serum from the same patients was further evaluated. For that, a strategy was developed based on the equalization of serum protein content (combinatorial peptide ligand library), followed by identification, by MALDI-TOF/TOF analysis, of the glycoproteins carrying the truncated glycans by 2D Western blot using monoclonal antibodies against these glycan antigens. Some of the identified glycoproteins seem to be promising since they have been reported to play a role in *H. pylori* chronic infection of the gastric mucosa and in cancer cell invasion by interacting with extracellular matrix (ECM). The identified glycoproteins are being validated as markers of gastric disease and their role in gastric cancer development is under study. This work was funded by FCT PIC/IC/82716/2007. CG thanks FCT for a PhD grant.

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172: Release of alkaline phosphatase caused by PIGV mutations in patients with Hyperphosphatasia-Mental Retardation syndrome (HPMR), a recently found second inherited GPI anchor deficiency

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Whole exome sequencing of sibs of non-consanguineous parents demonstrated PIGV mutations in Hyperphosphatasia-Mental Retardation syndrome (HPMR), an autosomal recessive disease characterized by mental retardation and elevated serum alkaline phosphatase (ALP) levels (Nature Genet. Vol.42 p827 2010). PIGV is the second mannosyltransferase essential for glycosylphosphatidylinositol (GPI) biosynthesis. Mutations found in four families caused amino acid substitutions A341E, A341V, Q256K and H385P. We have shown that these mutant PIGV proteins are unstable and the mutant cDNAs restored only subnormal GPI biosynthetic activity after transfection into PIGV deficient CHO cells. GPI-anchor is synthesized in the endoplasmic reticulum (ER) and is transferred to the proteins which have GPI attachment signal at the C-terminal. GPI-anchored proteins are not expressed on the surface of GPI deficient cells due to degradation within the cells or secretion. ALP, a GPI anchored protein, was efficiently secreted into medium from PIGV deficient CHO cells, in which incomplete GPI bearing one mannose was accumulated. In contrast, ALP was degraded in PIGL, DPM2 or PIGX deficient CHO cells, in which GPIs lacking mannose were accumulated. Secretion of ALP

required GPI transamidase that cleaves the C-terminal GPI attachment signal peptide and replaces it with GPI. It seems that GPI transamidase is activated by GPI bearing at least one mannose, cleaving the hydrophobic signal peptide and resulting in secretion of soluble ALP.

It is well known that hypophosphatasia is caused by a deficiency of the tissue-nonspecific alkaline phosphatase (TNAP). Some patients with this inherited disorder develop epilepsy caused by the disturbance of pyridoxal-5-phosphate (PLP) dependent metabolism of neurotransmitters due to the defect in TNAP. Some of the HPMR patients have seizures and the patients with previously reported inherited GPI deficiency caused by defective PIGM encoding the first mannosyltransferase (Nature Med. 12, p846 2006) also have seizures. This common symptom may be caused by the loss of membrane bound TNAP.

173: Conjugate vaccine against *Clostridium difficile*

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Clostridium difficile is a Gram-positive bacterium causing enteric diseases in many animal species. In humans, *C. difficile* associated diarrhea (CDAD) is the most commonly diagnosed cause of hospital-associated and antimicrobial-associated diarrhea. Several recent outbreaks of more virulent strains have led to epidemics in North America and Europe [1, 2]. At the moment, there is no vaccine against *C. difficile*, and the prophylactic use of antibiotics to prevent the *C. difficile* infection has led to an increase in the incidence of the disease. *Clostridium difficile* strain produces three main toxins toxin A, toxin B and CDT (binary toxin) and express two highly complex cell-surface polysaccharide PS-I and PS-II. The polysaccharide PS-II, composed of a hexaglycosyl phosphate repeating unit [-6)-β-D-Glcp-(1-3)-β-D-GalpNAc-(1-4)-α-D-Glcp-(1-4)-[β-D-Glcp-(1-3)]-β-D-GalpNAc-(1-3)-α-D-Manp-(1-P], is present in several strains and appears to be a common *C. difficile* polysaccharide and thus it may be advantageous in the development of a carbohydrate-based anti-*C. difficile* vaccine [3]. Several strains were observed to produce the PS-II polysaccharide by NMR carried out on intact cells, and purification of PS-II was developed using a best yielding strain. After selective modification to generate aldehyde groups, PS-II was coupled to CRM197 via reductive amination. Structural characterization of the purified polysaccharide and glycoconjugates was carried out using physicochemical methods (NMR, Amino Acid Assay, HPAEC-PAD, HPLC, MicroBCA, SDS-Page). Groups of 8 CD1 mice were injected i.p. with 2.5 μg of conjugate (saccharide base) with Alum and MF59 as adjuvants at 0, 21, 35 days. Bleeding performed at 0, 34, 49 days. Mice sera were tested for presence of anti PS-II antibodies using an ELISA procedure and the results showed that the conjugates were able to induce high anti PS-II IgG titers.

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174: Identification of important biological Glyco-epitopes of Lubricin in Synovial Fluid

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Rheumatoid arthritis (RA) is a chronic, inflammatory, autoimmune disease. It primarily affects the joints in a symmetric pattern. A particular feature of RA is inflammation and soft-tissue swelling of many joints at one and the same time. The joints are dramatically affected that cause a tremendous amount of pain, swelling and loss of motion to those affected. Since the glycosylation pathway is also regulated during inflammation, there is need to find novel glyco-epitopes of glycoprotein like molecule in synovial fluid that may serve as a potential biomarker for diagnosing chronic articular inflammation. Lubricin (Proteoglycan 4) is the dominating component among the acidic glycoproteins in synovial fluid responsible for lubricating the synovial joints which is the primary organ for symptoms of RA. Human synovial lubricin (1404 amino acids), a mucin like molecule, is encoded by PRG4 and synthesized in synovial fibroblast and synovial chondrocytes. The central mucin like domain is characterized with 59 perfectly/imperfectly repeating units of EPATTPK. Heavily O-glycosylated mucin like domain gives lubricin boundary lubricating ability. As glycosylation is regulated during inflammation, novel glyco-epitopes will emerge leading to excessive interaction with glyco-epitope binding protein that can facilitate inflammation. Therefore, these new glyco-epitopes of lubricin may serve as a potential biomarker for advanced diagnosis. We used various biotinylated lectin and anti-carbohydrate antibodies to characterize glycosylation regulations in inflammation of lubricin. Lectins include PNA, WGA, SNA, HAA, AAL, MAL, and anti-carbohydrates antibodies were specific to T antigen, Tn, sialyl-Tn, sialyl lewis x/a, lewis b, MECA-79 and chondroitin sulfate. The results indicate that lubricin contains immunologically important O-liked oligosaccharide

epitopes that may serve as potential biomarkers for diagnosing chronic articular inflammation.

175: Determination of glycosyltransferase genes involved in the biosynthesis of Sd^a and sLex epitopes in normal and cancer gastrointestinal cell lines.

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The Sd^a/Cad antigen (GalNAcβ1-4[Neu5Acα2-3]Galβ1-4GlcNAc), is an onco-developmentally regulated antigen expressed on glycolipids and glycoproteins in the human gastrointestinal (GI) mucosa with an increasing gradient from the ileum to the colon. On the other hand, the sLe^x antigen (Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAc) is not or very weakly expressed on human GI glycoproteins. In GI cancer, the expression of sLe^x is strongly increased whereas Sd^a expression is deeply decreased, suggesting a balanced expression of Sd^a and the sLe^x antigens (Malagolini et al., 2007). The molecular basis of sLe^x overexpression at the expense of Sd^a antigen in GI cancer is still unclear. We have analyzed the expression of the sLe^x and Sd^a/Cad antigens using Immunocytochemistry (FACS analysis) and western-blotting in normal and cancer GI cells (Colo 205, HT29, Caco-2, MKN45) and other control cells (HeLa and HEK293). We have investigated whether expression of the Sd^a epitope could be linked to the transcriptional expression of several glycosyltransferases involved in its synthesis. Q-PCR approach has been carried out to analyze the transcriptional expression of (i) the 3 sialyltransferase genes (ST3GAL3, ST3GAL4, ST3GAL6), (ii) the 5 fucosyltransferase genes (FUT3, FUT4, FUT5, FUT6, FUT7) potentially involved in sLe^x biosynthesis and (iii) the two B4GALNT2 transcripts involved in Sd^a biosynthesis (Montiel et al. 2003).

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176: Binding affinities of Yariv reagents to lectin PA-IL from *Pseudomonas aeruginosa*

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Different Yariv phloroglucinol derivatives have been synthesized. These Yariv reagents are trivalent glycoconjugates and some of them are characterized by their ability to bind specifically to arabinogalactan-proteins from plants. In this study, Yariv phenylglycosides have been evaluated as ligands for the galactose-binding lectin PA-IL from the opportunistic bacterium *Pseudomonas aeruginosa*. Investigations by isothermal titration calorimetry [1] revealed that Gal-Yariv reagent shows binding affinity to PA-IL. Using a freshly prepared solution of Gal-Yariv reagent in a concentration of 0.33 mM, titration with PA-IL (0.1 mM) resulted in binding of three Gal-residues to the lectin. Interestingly, binding properties decreased after a few hours, maybe due to stacking of Yariv-reagents in solution. Additionally, binding capacities varied depending on concentrations of ligands and lectin. Surface plasmon resonance experiments [2] confirmed that the Gal-Yariv reagents are inhibitors of PA-IL. References: 1. Nurisso A et al. (2010) J Biol Chem 285: 20316-20327; 2. Cecioni S et al. (2009) Chem Eur J 15: 13232-13240.

177: New sugar marker attached to PSA for prostate cancer

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Prostate-specific antigen (PSA) is recognized as the premier tumor marker for prostate cancer, the incidence of which continues to rise in the most regions of the world. However, the false positivity on the basis of serum PSA contents in the gray zone should be diminished for the clear distinction between prostate cancer (PC) and benign prostatic hyperplasia (BPH). Since the structural changes of N-glycans during carcinogenesis are common phenomena, we investigated whether PC-specific N-glycans are linked to PSA. First, the carbohydrate structures of PSA derived from seminal fluid, BPH serum, PC serum and PC cell line, LNCaP, were analyzed using eight lectin-immobilized column chromatographies followed with ELISA. We found the increase of *Trichosanthes japonica* agglutinin-II (TJA-II)-bound PSA in PC serum, not but in BPH serum, suggesting that α1,2-fucosylation and β-N-acetylgalactosaminylation of PSA elevated during carcinogenesis. It was confirmed that these carbohydrate structural changes were responsible for the enzyme fucosyltransferase I (FUT1) and β-N-acetylgalactosaminyltransferase 4(B4GALNT4) by real time PCR assay. Second, the contents of TJA-II bound PSA and binding ratios to TJA-II column chromatography were measured in 20 PC serum PSA and 20 BPH serum PSA, which of all were diagnosed histopathologically. Since PSA forms a covalent complex with serum a1-antichymotrypsin, the analysis of most carbohydrate structures on PSA should be accomplished by ELISA for free PSA. However, the Fucα1-2Gal and β-GalNAc residues are not involved in a1-antichymotrypsin, and can be detected by ELISA for total PSA. As a result, both the contents of TJA-II bound

PSA and the percentage of TJA-II binding could discriminate at over 95% probability between PC and BPH, and TJA-II bound PSA will become a promising improved marker of PC. Lectin-immobilized colomn chromatography and the following ELISA is very precise, but not applicable to diagnosis at the clinical spot. We are now developing a new diagnostic technology to discriminate prostate cancer with benign prostatic hyperplasia.

178: Correlation between low salivary sulfotransferase activities and clinical parameters in Sjögren's syndrome patients.

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Sjögren's syndrome (SS) is a chronic autoimmune disease of the exocrine glands. Altered secretory function leads to the symptoms of dry mouth and eyes. Salivary mucins are essential for lubrication of the oral epithelium. Mucins in the labial salivary gland (LSG) secretions from SS-patients have been shown to be undersulfated. The present study was addressed to determine the enzymatic basis for this phenomenon. We measured the expression and enzymatic activities of sulfotransferases and glycosyltransferases involved in mucin sulfation and correlated sulfotransferase activity with clinical parameters, including secretory function, inflammation and serology. LSG from 31 SS-patients and 31 control subjects were studied. Relative mRNA and protein levels of sulfotransferases (GAL3ST) and β3Gal-transferase-5 were determined by real-time RT-PCR and Western blotting, respectively. The levels of Gal3ST activity were significantly decreased in SS-patients without changes in mRNA and protein levels. In contrast, the activities of glycosyltransferases were similar in both groups. An inverse correlation was observed between Gal3ST activity and glandular function measured by scintigraphy, focus score, as well as with the autoantibodies Ro/SS-A and La/SS-B, but not with unstimulated salivary flow. The decrease in sulfotransferase activity may explain mucin hyposulfation observed in the LSGs from SS-patients. We postulate that pro-inflammatory cytokines induced by circulating autoantibodies may modulate Gal3ST activity, thereby altering mucin quality and leading to mouth dryness. The work was funded by Fondecyt 1080006 and the Canadian Cystic Fibrosis Foundation.

179: Withdrawn

180: Plasma membrane-associated sialidase (NEU3) regulates progression of prostate cancer through modulation of androgen receptor signaling

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Prostate cancers generally become androgen-independent and resistant to hormone therapy with progression. To understand underlying mechanisms and facilitate development of novel treatments for androgen-independent prostate cancer, we have investigated plasma membrane-associated sialidase (NEU3), the key enzyme for ganglioside hydrolysis participating in transmembrane signaling. We previously showed that NEU3 is up-regulated in various human cancers including colon, renal, and ovarian cancers, and contributes to expression of a malignant properties including suppression of apoptosis and promotion of cell invasion and motility. Here we have discovered NEU3 to be up-regulated also in human prostate cancer compared to noncancerous tissue, correlating with the Gleason score. NEU3 silencing with siRNA in prostate cancer PC-3 and LNCaP cells resulted in increased expression of differentiation markers and in cell apoptosis, but decrease in Bcl-2 as well as a progression-related transcription factor, early growth response gene 1 (EGR-1). In androgen-sensitive LNCaP cells, forced overexpression of NEU3 significantly induced expression of EGR-1, androgen receptor and prostate specific antigen both in the presence and absence of androgen, the cells becoming sensitive to androgen. The NEU3-mediated induction was abrogated by specific inhibitors for PI-3 kinase and MAPK in the absence of androgen, being confirmed by increased phosphorylation of AKT and ERK1/2 in NEU3-overexpressing cells. NEU3 siRNA introduction caused reduction of cell growth of an androgen-independent PC-3 cells in culture and of transplanted tumors in nude mice. These data suggest that NEU3 regulates tumor progression through androgen receptor signaling and thus be a potential tool for diagnosis and therapy of androgen-independent prostate cancer.

181: Transcriptional regulation of FUT4 in breast cancer epithelial cells

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Lewis Y (Le^y) is a carbohydrate tumor-asssociated antigen. The majority of cancer cells derived from epithelial tissue express Le^y type difucosylated oligosaccharide. Fucosyltransferase IV (FUT4) is an essential enzyme that

catalyzes the synthesis of LeY oligosaccharide. Our previous studies have shown that overexpression of fucosyltransferase IV promotes A431 cell proliferation through activating MAPK and PI3K/Akt signaling pathways. In this study, we investigated the transcriptional regulation of human FUT4 in human breast cancer cells (MCF-7, MDA-MB-231, MDA-MB-435, SUM1315, BT-549). Using a series of promoter deletion constructs, we indentified that a potential regulatory site that is located between 0.8-1.2kb of the FUT4 promoter. Electromobility shift assays (EMSA) suggested that sp1 was required for maximal promoter activity. Site-specific mutagenesis resulted in reduced protein binding. Furthermore, knock down of FUT4 decreased sp1-induced DNA synthesis and cell cycle progression, as well as the cell-cycle related regulatory proteins. These results suggest that FUT4 is a target gene for sp1 that is required for cell cycle progression in breast cancer epithelial cells.

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182: Glycobiology of human gastrointestinal mucins in pathophysiological conditions

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Gastrointestinal mucins are high molecular weight glycoproteins characterized by many O-linked oligosaccharides to the core polypeptide through Ser or Thr residues. These O-glycans have many diverse functions in biological systems. They are aiding in the conformation and stability of proteins and play a major role in defence of mucosae covering and protecting epithelium against various types of aggression. Profound alterations of mucin O-glycans are observed in diseases such as cancer, inflammation and bacterial infection, modifying the function of the cell and its antigenic and adhesive properties. Using tandem mass spectrometry and NMR techniques, we have established the repertoire of glycosylation of mucins isolated from different human adult and fetal tissues (stomach, small intestine, colon). We have demonstrated that region-specific glycosylation of mucins along the intestinal tract is acquired after birth, probably due to bacterial colonization and gut postnatal absorptive and digestive functions. Using human tissues as well as animal models, we have also demonstrated important modification of expression, localisation and glycosylation of mucins in pathological conditions such as colorectal cancer, bacterial infection (*Shigella flexneri*), inflammation and chronic psychological stress. All these studies allow to better understand relation structure/functions and to propose the use of certain mucins and/or particular O-glycans as potential biomarkers useful in the prediction, prognosis or diagnostic of intestinal diseases.

183: Anti-glycan antibodies in plasma of patients with colorectal cancer

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Colorectal cancer is the third leading cause of cancer-related death in the Western world with 655,000 deaths worldwide per year. Invasive cancers that are confined within the wall of the colon (TNM stages I and II) are curable with surgery, but if untreated, they spread to regional lymph nodes (stage III), and distant sites (stage IV) what is associated with poor prognosis. Since early diagnosis is essential for successful treatment, development of reliable screening tools is of utmost importance. Glycans are predominant surface components of eukaryotic cells and microorganisms. As such, they give rise to high levels of anti-glycan antibodies of all classes. Apart, so called natural antibodies to certain defined mono, di and oligosaccharides, common in bacterial, fungal and parasite cells, are pre-exist in human sera and can be profiled using glycan arrays. Alterations in metabolism of cancer cells result in the production of altered glycan structures, which are being recognized by the immune system and result in generation of novel anti-glycan antibodies. Using printed glycan array with preliminary chosen 48 glycan structures we screened 193 control samples from healthy donors and 197 samples of plasma from patients with CRC. Our results show that plasma anti-glycan antibodies from both healthy donors and CRC patients can bind up to 75 % of total number of plasma samples in each group, but in case of CRC the binding to some glycans is found to be more frequent.

184: High throughput isolation and glycosylation analysis of IgG – variability and heritability of the IgG glycome in two isolated human populations

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All immunoglobulin G molecules carry N-glycans which modulate their biological activity. Changes in N-glycosylation of IgG associate with various diseases and affect the activity of therapeutic antibodies and intravenous immunoglobulins. We have developed a novel 96 well protein G monolithic plate and used it to rapidly isolate IgG from plasma of 1821 individuals from two isolated human populations. N-glycans were released by PNGase F, labeled with 2-aminobenzamide and analyzed by hydrophilic interaction chromatography with fluorescence detection. The majority of the structural features of the IgG glycome were consistent with previous studies, but sialylation was somewhat higher than reported previously. Sialylation was particularly prominent in core-fucosylated glycans containing two galactose residues and bisecting GlcNAc where median sialylation level was nearly 80%. Very high variability between individuals was observed, approximately three times higher than in the total plasma glycome. For example, neutral IgG glycans without core fucose varied between 1.4% and 19%, a difference that significantly affects the effector functions of natural antibodies, predisposing or protecting individuals from particular diseases. Heritability of IgG glycans was generally between 30% and 50%. Individual's age was associated with a significant decrease in galactose and increase of bisecting GlcNAc, while other functional elements of IgG glycosylation did not change much with age. Gender was not an important predictor for any IgG glycan. An important observation is that competition between glycosyltransferases which occurs in vitro did not appear to be relevant in vivo, indicating that the final glycan structures are not a simple result of competing enzymatic activities, but a carefully regulated outcome designed to meet the prevailing physiological needs.

185: Withdrawn

186: Metal Ion Dysregulation in Lysosomal Storage Diseases

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Lysosomal storage diseases (LSDs) are a group of disorders, which have defective lysosomal function, resulting in the accumulation of macromolecules in the lysosomal system. In this study, we have investigated ion regulation in a mouse model of Niemann Pick type C1 (NPC1) disease and found evidence of altered metal ion homeostasis. Using SDS-PAGE/immunoblotting, we found that several key regulators, which are involved in iron/copper homeostasis, have altered protein expression patterns, including transferrin receptor (Tf-R), transferrin (Tf), ferritin, and ceruloplasmin (Cp; a copper containing protein), in brain, liver, and serum in the NPC1-/- mouse. Also, using native PAGE/immunoblotting, we detected different levels of ferritin complexes. Furthermore, altered level of transferrin receptor (Tf-R) was detected in brain, liver, spleen, kidney, serum, and cultured NPC1-/- glial cells. In addition, non-heme iron assay showed total serum iron (Fe3+ and Fe2+) and ferrous ion (Fe2+ only) levels from end stage NPC1-/- mice were significantly lower when compared with wild type controls. These data suggest that iron/copper dysregulation occurs in NPC1 disease mice, as well as in mouse models of GM1 and GM2 gangliosidoses, as we previously reported [1]. Since metal ions are essential for metabolic processes and can cause pathology when dysregulated, our data suggest that altered metal ion homeostasis may be involved in the pathogenesis of lysosomal storage diseases. On the basis of these findings, we aim to investigate the mechanism of ion dysregulation and explore the potential of ion-related biomarkers in LSDs.

[1] Jeyakumar M, Williams I, Smith DA, Cox TM, Platt FM. Critical role of iron in the pathogenesis of the murine gangliosidoses. Neurobiology of Disease 2009;34(3):406-416.

Poster Session I (Monday)

Cell biology

187: Suggestion of the existence of endogenous sialidase on a cell surface of the mouse thymus analyzed by FACS

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The crude membrane fraction of the mouse thymus contains a high level of sialidase activity measured with 4MU-Neu5Ac as a substrate at neutral pH compared to those of the spleen and lymph nodes. This activity showed an independent peak at pH 6.5-7.0 [1]. We have cloned the cytosolic soluble Neu2 gene from the mouse thymus [3] and we have transfected the plasmid vector into COS cells

and shown the existence of membrane-bound Neu2 sialidase in COS cells [4]. We found that the SM (Neu1[a]) mouse thymus lacks Neu2 activity because of a low expression level [2].

FACS analysis of single cells from the thymus suggested the existence of an endogenous sialidase on the cell surface. Unadhered cells on a PNA (pea nuts agglutinin)-coated dish from the thymus (maturated T cell-rich) were stained much more with PNA after incubation with thymus sialidase or even with PBS alone. We tried to explain the existence of endogenous sialidase on the cell surface by histochemical data for Neu2-transfected COS cells [4]. The sialidase-positive cells in the thymus were also characterized by antibody-coupled bead enrichment. Furthermore, we found the existence of membrane-bound sialidase that has high activity at pH 7.0 and very low activity at pH 4.5 by isoelectric separation of the NP40-solubilized fraction from thymus crude membranes, and this enzyme had an isoelectric pH that was the same as or slightly higher than that of cytosolic Neu2 and might be regulated by the Neu2 gene as suggested by analysis of the activity of SMXA recombinant inbred mice. An immuno-precipitation experiment showed that a part of the Neu2 enzyme was a membrane-bound form.

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188: Extremely low expression of cytosolic sialidase in human tissues and its probable involvement in cell survival of a human cancer cell line

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Cytosolic sialidase Neu2 was the first example of a mammalian sialidase for which cDNA cloning was achieved from rat skeletal muscle (1). The human homologue NEU2 have so far been studied using NEU2 cDNA constructed from a genomic library of human skeletal muscle (2), and its three-dimensional structure has been determined by X-ray crystallography (3). However, NEU2 expression level has not been clearly determined in human tissues and cells. In the present study, we have obtained a cDNA using poly(A)+RNA from human brain and colon, and investigated whether this sialidase is actually expressed and what roles it plays if exists. Compared with other three sialidases, NEU2 expression was extremely low and undetectable in most of human tissues and cells examined. NEU1 was the highest expression, 10-20 times higher than those of NEU3 and NEU4, while NEU2 expression was extremely low, only four- to ten- thousandth of the NEU1 value at the most in a wide range of tissues, as assessed by quantitative real time RT-PCR using a standard curve for each cDNA. The cDNA obtained demonstrated sialidase activity towards oligosaccharides, glycoproteins and gangliosides as substrates, with enzymatic properties similar to the murine ortholog. The expression was at a detectable level in several human tissues, especially, in placenta and testes. Unexpectedly, prostate cancer PC-3 cells showed relatively high NEU2 expression. In NEU2-overexpressing and -silencing experiments in the cells, it is likely to be involved in cell survival and cell motility.

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(2) Monti E, et al. (1999) Genomics. 57,137-143.
(3) Chavas, L. M., et al. (2005,) J. Biol. Chem. 280, 469-475.

189: JM403 antigen GlcA-GlcNH3+ on heparan sulfate glycosaminoglycan may be involved in cell proliferation and apoptosis

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Compared with studies on their core proteins, the studies about the glycans of heparan sulfate proteoglycans (HSPGs), called HS glycosaminoglycans (HSGAGs), in human cancers are quite limited. The myriad of the heterogeneity of HSGAGs caused by the differences of glycan-lengths, sulfations and epimerization has hindered analyses of HSGAGs from small amounts of biological materials, even though current glycomics techniques are available. However, since HSPGs are crucial molecules to affect on cancer cell behaviors through signal transductions, it is urgently needed to examine the HSGAGs from cancer cells. Using immunohistochemistry (IHC) with monoclonal antibodies, we investigate whether or not specific structures of HSGAGs promote cancer cell proliferation. Although all HSGAGs antigens were variously detected on the surface of human breast cancers, unexpectedly they were also detected in the cytoplasm with granular patterns. Among them, the JM403 antigen (GlcA-$GlcNH_3^+$) was most frequently observed (about 60% cases were positive) and well correlated to the conventional malignant parameters of Ki67 labeling index and mitotic counts, suggesting that JM403 antigen might be involved in cell proliferation. IHC study, using human small intestines,

revealed the co-expression of the JM403 antigens in cytoplasms at the proliferative compartment with large granular pattern, together with Ki67 protein in the nucleus in the same cells. On the other hand, at the top of the villi, where casepase-3 and TUNEL stainings were positive, the JM403 antigens were detected along with cell surface membranes with fine granular pattern. Furthermore, flow cytometry technique revealed an increase level of the JM403 antigen on the cell surfaces of the breast cancer cells as well as many other cancer cells, when apoptosis was induced. Importantly, these increases were always observed regardless of types of stimuli that induced apoptosis. These results suggest that the JM403 antigens may involve not only in cell proliferation, but also in apoptosis.

190: Biological roles of chondroitin sulphate in cutaneous wound healing

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Chondroitin sulphate is a long, unbranched, highly polyanionic glycosaminoglycan, and forms an important constituent of skin, connective tissue, cartilage and bone. Besides its structural function, chondroitin sulphate has been reported to bind to and interact with various growth factors and signalling molecules, thereby regulating cellular behaviour. The specificities of these molecular interactions are dependent on the presence of sulphate groups on the chondroitin chain. Skin wounds are one of the most common injuries in life. Delayed wound healing is often seen in patients with poorly controlled diabetes mellitus. To better understand the biological roles of chondroitin sulphate in wound healing, we created full thickness skin wounds in Sprague Dawley rats and applied various chondroitin sulphate species to the wound. Our results showed that the number and position of the sulphate groups on the chondroitin molecule influences the ability of the molecule to promote skin wound healing. Further, the different chondroitin sulphate species have varying effects on deposition of collagen in the healing wound as well as the formation of new blood vessels. We also carried out complementary experiments using an in vitro wound healing model. Through the use of siRNA, we perturbed the biosynthesis of chondroitin sulphate, and found that this resulted in a reduction in cell migration. Cell proliferation was not affected. These results suggest that chondroitin sulphate is of fundamental importance in wound healing, and holds promise as a therapeutic target.

191: Analysis of carbohydrate specificity of C-type lectin receptors as antigen-uptake receptors using neoglycolipids-coated liposomes

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Pathogen recognition and uptake by antigen-presenting cells (APCs) and subsequent presentation of antigen on MHC molecules to activate T cells are crucial in the induction of adaptive immunity. The C-type lectin receptors (CLRs) expressed on APCs are particularly important for these processes, and therefore, CLRs are recognized as antigen-uptake receptors. We have shown that the liposomes coated with a neoglycolipid with the mannotriose residue (Man3-DPPE) are preferentially taken up by resident peritoneal macrophages (RPMs), leading antigen-specific Th1 immunity. RPMs express SIGNR1 and SIGNR3, which belong to mannose-binding CLR family. Although these CLRs are shown to recognize similar carbohydrate structures such as high-mannose containing structures and Lewis antigens, our preliminary experiments indicate that SIGNR1 but not SIGNR3 serves as a receptor for the oligomannose-coated liposomes. To understand the carbohydrate preferences of CLRs as antigen-uptake receptors, in the present study, we analyzed the uptake of neoglycolipid-coated liposomes by the CLRs. The FITC-BSA encased liposomes coated with various types of neoglycolipids were added to CHO cells transiently expressed FLAG-tagged CLRs molecules, and the cells were stained with anti-FLAG antibody. SIGNR1-expressing cells take up Man3-DPPE coated liposomes much more effectively than SIGNR3- or Langerin-expressing cells, although all types of cells could ingest zymosan almost similarly. When uptake of Man2-, Man3-, and Man5-DPPE coated liposomes were tested, Man3-DPPE-coated liposomes were most effectively incorporated into the cells among these neoglycolipids-coated liposomes. LNFP-III- and BNCP-DPPE coated liposomes were also ingested in the cells, but the uptake efficiency of these liposomes seemed to be lower than that of Man3-DPPE coated liposomes. In addition, we found that uptake preferences of neoglycolipids-coated liposomes of SIGNR-3 and Langerin differ from that of SIGNR1. Interestingly, the levels of antigen-specific IFN-gamma production from splenocytes induced by immunization of antigen-encased neoglycolipids-coated liposomes are similar to the preference of liposomal uptake by SIGNR1. Therefore, efficiency of uptake of carbohydrate-coated liposomes by APCs through CLRs may determine the levels of subsequent immune responses induced by the liposomes.

192: Lectins and enzymes: universal intramolecular cooperation in organisms

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Historically, examples of lectin-enzyme relationships were described as intermolecular and then intramolecular type. The aim was to represent and describe examples of both type co-functioning lectins and enzymes. On the one hand, about 500 enzymes (also their protein non-lectin type modulators) according to International Enzyme Classification are described as real targets for extended panel of carbohydrate sensitive agents (mainly exogenic non-mammalian and endogenic mammalian biotope lectins)[1]. On the other hand, at present there are a lot of examples when oxidoreductases, transferases, esterases, glycosyl hydrolases, peptidyl hydrolases, amidases, lyases, isomerases, and ligases act as true lectins, and they are organized as molecular lectin and enzyme compartments. On the one hand, own human interactome (higher hierarchy) proposes a complex net of cascade or non-cascade inter- and intra co-functioning own lectins and enzymes. On the other hand, microbiocenoses (lower hierarchy) in human organism functions as a part of superorganism. The majority of ways of lectin-enzyme functioning of both hierarchies are invisible to each other (independence as a key of human survivial). On the one hand, probiotic lectin-enzyme cooperation is really useful for the human host. On the other hand, pathogen lectin-enzyme cooperation can act as virulent factors. Probiotics possess a number of enzyme-lectin mechanisms against pathogens or their pathogenic actions. Among possibilities, increasing probiotic anti-pathogen protection system in human body is the perspective one. (1) Lakhtin M, Lakhtin V, Afanasyev S, Alyoshkin V, and Alyoshkin A (2010) [Lectins and enzymes in biology and medicine]. Moscow: Dynasty, 2010. 496 pp. In Russian

193: Galectin-8 as a novel lectin modulating cancer metastasis

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Galectin-8 is a mammalian lectin, which specifically binds beta-galactosides containing glycoconjugates. It is highly expressed and secreted by prostate cancer both invasive or in early stages, suggesting that secretion of galectin-8 may aid in the progression of prostate cancer metastasis and the settlement of the metastatic lesion. Microarray analysis carried out in our lab demonstrated the ability of galectin-8 to alter transcription of several genes relevant to cell transformation and metastasis. For example, galectin-8 elevated the expression of BMP2A (bone morphogenetic protein 2A) and uPA (urokinase-type plasminogen activator), two major factors promoting metastasis of prostate cancer cells. It was further discovered that PC3 cells (human prostate adenocarcinoma cells derived from a bone metastatic site) express a longer splice isoform of galectin-8 (G8L), which is less common than the major isoform (G8M). The ratio of G8L to G8M expression was higher in PC3 cells compared to prostate cell lines derived from a metastatic lesion in the lymph node or the primary tissue. In PC3 cells both isoforms elevated the expression levels of MMP9 (Matrix metallopeptidase 9), an important mediator of metastasis, as well as BMP2A and uPA, while G8L had a higher effect compared to G8M at the same concentrations. The overexpression of G8L, together with its ability to further elevate the expression of genes related to metastasis, may be part of the mechanism by which prostate cancer cells perform metastasis.

194: CLEC-2 is a highly conservative C-type lectin-like receptor of Dectin-1 cluster and the expression is regulated by different mechanisms in evolution

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CLEC-2 was first identified by sequence similarity to C-type lectin-like molecules with immune functions and has been reported as a receptor for the platelet-aggregating snake venom toxin rhodocytin and the endogenous sialoglycoprotein podoplanin. Recent reports indicate that CLEC-2 has a role in the regulation of embryonic vascular development and CLEC-2-deficient mice exhibit abnormal patterns of lymphatic vessel formation, which suggests that CLEC-2 might have a unique role in the individual development. In this study, we reported that CLEC-2 from different species holds with an extraordinary conservation on sequence alignment. Furthermore, some functional structure such as N-linked oligosaccharides and the ITAM motif have a similar role in a variety of species. Nevertheless, isoforms of CLEC-2 generated by alternative splicing, a regulatory mechanism of gene expression and the binding sites for several key transcription factors vary between different species suggesting that the expression of CLEC-2 is tightly regulated by different mechanisms in different species.

195: Neoglycolipids as a tool for study of cellular ligands of galectins

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Identification of cellular ligands of glycan-binding proteins is the key for understanding of their biological functions. We believe that insertion of neoglycolipids into cell membrane following galectin loading allows modifying the anchoring events. We synthesized a series

of neoglycolipids where glyco part is known as galectin ligands, namely Le^c-sp-PE, LN_2-sp-PE, H (type 2)-sp-PE, A (type 2)-sp-PE, Galß1-3GalNAcß1-4Galß1-4Glc-sp-PE (GA1) and 3'SiaLac-sp-PE, by condensation of ω-aminoalkyl glycosides with phosphatidylethanolamine (PE). The kinetics of insertion of H (type 2)-sp-PE, GA1-sp-PE and 3'SiaLac-sp-PE comparing with natural asialoGM1 and GM1 into Raji (human B-lymphocytes) and L-929 (mouse fibroblasts) cells was studied. It was shown that independently of cell type natural and neoglycolipids inserted into cell membrane reached the maximum value in 1 h, and did not internalize into cells for 24 h. Desialylation followed by neoglycolipids insertion led to their internalization in cytoplasm for 1 h. Further we modified Raji cells by neoglycolipids - ligands of galectins, and studied how the insertion affected galectins (kindly provided by Prof. H.-J. Gabius, Munich, Germany) anchoring. It was shown that modification of Raji cells with LN_2 led to immersion of human galectin-1 (but not galectin-3) into glycocalix. Besides, insertion of LN_2 or 3'SiaLac (but not A (type 2)) provided increasing of immersion of tandem type galectin-9N in glycocalyx, whereas did not affect the binding of galectin-8N. Thus, the change of glycosylation pattern with the help of synthetic glycolipids seems to be a useful method for studying galectin anchoring on cell membrane (The work is supported by the grant of Russian Foundation for Basic Research N–10-04-00959).

196: Protective effect of gangliosides and heme oxygenase in obstructive cholestasis

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Aims: Bile acids have been implicated in the cholestatic liver damage by mechanisms involving detergent effect on cellular membranes and oxidative stress. Gangliosides can counteract the detergent effect of bile acids by increasing the rigidity in the outer layer of the plasma membrane. Induction of heme oxygenase-1 (HO-1), the rate limiting enzyme in heme catabolic pathway, has been shown to protect liver against oxidative stress. The objective of this study was to examine expression and supposable redistribution of GM1 ganglioside and to study possible influence of HO-1 modulation on GM1 expression and localization. Methods: Wistar rats were pretreated with hemin (HO-1 induction) or Sn-mesoporphyrin (HO-1 inhibition) followed by bile duct ligation (BDL,5 days) or sham operation (each group n=6). Area of ductular proliferation caused by obstructive cholestasis was measured in liver sections. GM1 was detected using cholera toxin B-subunit. Image analysis was used for evaluation of GM1. Expression of galactosyltransferase 2 (GalT2),the key enzyme in GM1 synthesis, was evaluated. Results: Compared to controls, BDL, as well as HO-1 inhibition, resulted in higher ductular proliferation (436% and 300%,respectively,$P<0.01$) compared to controls, while HO-1 activation significantly decreased the proliferation in BDL rats (83%,$P<0.05$). Marked shift of GM1 expression from cytoplasm to plasma membranes has been observed in BDL rats. An additional enhancement of membrane expression was in group with inhibited HO-1 and fall of the expression in group with activated HO-1. Moreover, HO-1 inhibition resulted in a significant increase in GalT2 expression compared to controls (184%,$P<0.01$), while HO-1 induction had an opposite effect (39%,$P<0.05$). Conclusions: Close relationship between HO-1 activity and expression of GalT2 was observed. While HO-1 induction has a hepatoprotective effect and is accompanied by decreased ductular proliferation and ganglioside synthesis in the liver of BDL rats, HO-1 inhibition leads to more pronounced liver injury with increased GalT2 activity and compensatory membrane redistribution of gangliosides. Supported by grants IGA-MZ-11327-4, GAUK-251207.

197: Immunohistochemical Detection of Glycolipids in Various Tissues of Rat

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Localization of glycolipids in various tissues of rat was investigated by using confocal laser scanning microscopy and transmission electron microscopy. Wistar rats were prefixed by perfusion with 4% formaldehyde or 2.5% glutaraldehyde, and various organs (cerebellum, intestine, kidney etc.) were cut into nonfrozen slices (40 micronmeter thick). Sections were treated with monoclonal antibodies against glycolipids (anti-GD2, GD3, GT1b, GQ1b, Gb3 etc.) followed by treatment with Cy3-labeled secondary antibody for light microscopy or peroxidase-labeled secondary antibody for electron microscopy. In cerebellar cortex, each ganglioside revealed specific distribution pattern, e.g. anti-GQ1b antibody was positive for the plasma membrane of Purkinje cell body and its dendrites, Golgi cisternae, part of the nerve endings around the Purkinje cell body. In the case of small intestine, accumulations of some gangliosides (GD2, GQ1b) were observed around the nerve cell body in the myenteric plexsus and also along the plasma membranes of inner-circular smooth muscle cells. Intestinal gland epithelial cells, especially their basolateral plasma membranes were positive for Gb3 globoside. In the case of kidney cortex, each antibody revealed specific distribution pattern especially for the glomerular basement membranes and

for the glomerular and urinary tubular epithelial cells. For example, Gb3 was demonstrated to localize along the plasma membranes of podocyte foot processes. When the frozen sections were applied, staining intensities were significangly decreased indicating the unstable nature of the glycolipid antigens against the conventional freeze-thawing procedures. The methods employed in the present study overcome technical difficulties to visualize glycolipids for both light and electron microscopic levels.

198: Convergence and enhancement of the growth and adhesion signals by ganglioside GD3 in human melanomas.

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Ganglioside GD3 is widely expressed in human melanoma cells. In order to clarify the role of GD3 in malignant phenotypes in melanomas, we established GD3-expressing cells (GD3+) from a GD3-lacking mutant subline, SK-MEL-28 N1(N1). The GD3+ cells show stronger phosphorylation of MAPK, Akt, focal adhesion kinase (FAK), p130Cas and paxillin when treated with FCS than control cells. In this study, we analyzed integrins and integrin-linked kinase (ILK)/Akt signaling pathway during adhesion to collagen type I (CL-I). Silencing of integrin beta 1 or Akt in GD3+ cells resulted in suppression of proliferation and invasion activity suggesting that these molecules are essential for the tumor properties in GD3+ cells. The GD3+ cells showed stronger phosphorylation of Akt, FAK and paxillin during adhesion to CL-I. In the lipid rafts, a high level of integrin beta 1 was found in GD3+ cells before adhesion, and a significant level of phosphorylated FAK was also found in the lipid rafts of GD3+ cells during adhesion, while integrin and FAK were scarcely detected in the lipid rafts in control cells. These results suggested that integrins assembled and formed a cluster in the lipid raft, leading to the enhanced signaling and malignant properties under GD3 expression. ILK/Akt signal was also activated during adhesion more strongly in GD3+ cells than in controls. Knockdown of ILK revealed Akt phosphorylation was induced via integrin-ILK signals. Now, we are investigating the cooperative effects of signals mediated by growth factor receptors such as c-Met (HGF receptor) and those via adhesion on the tumor properties in GD3+ cells with focus on the convergence and enhancement of them comparing with individual single signals.

199: The role of UDP-galactose:ceramide galactosyltransferase (UGT8), new molecular marker of breast cancer malignancy and lung metastasis, in tumor progression

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The UDP-galactose:ceramide galactosyltransferase (UGT8) is the enzyme responsible for the synthesis of galactosylceramide (GalCer) which is the simple glycosphingolipid known mostly as one of the major compounds of myelin. Our studies on the expression of UGT8 in breast cancer tissue specimens showed that increased amounts of this enzyme in cancerous tissue are associated with progression to a more malignant phenotype. We analyzed also the presence of UGT8 and GalCer in breast cancer cells and found that cells with the 'luminal epithelial-like' phenotype did not expressed or weakly expressed UGT8 and GalCer, in contrast to malignant, 'mesenchymal-like' cells forming metastases in nude mice. Very little is known about the possible functions of UGT8 and GalCer in tumor cells, however it was proposed recently that accumulation of GalCer in tumor cells inhibits apoptosis, which facilitates metastatic cells to survive in the hostile micoenvironment of the target organ. To verify this hypothesis, human breast cancer MCF7 cells were transfected with UGT8 mRNA, and for induction of apoptosis in cells overexpressing GalCer as well as parental cells, they were grown in the presence of 2 μM of N-(4-hydroxyphenyl)retinamide (4-HPR) for 96 h. Apoptosis was evaluated by subjecting the cells to annexin V binding assay and western blotting for detection of active caspase 3. It was found that accumulation of GalCer increased the resistance of MCF7 cells to drug-induced apoptosis. Similar results were obtained, when MDA-MB-231 cells with stable expression of shRNA directed against UGT8 mRNA and characterized by decreased expression of GalCer were treated with 2.5 μM of doxorubicin for 48 h. In this case the down-regulation of UGT8 resulted in increase sensitivity of breast cancer cells to drug-induced apoptosis. In conclusion, we showed that accumulation of GalCer in breast cancer cell effectively inhibits the drug-induced apoptosis.

200: Withdrawn

201: A novel mannose-6-phosphate receptor homology (MRH) domain containing protein is involved in plant ER-associated degradation of proteins

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To avoid aggregation and cell damage, unfolded and terminally misfolded proteins in the endoplasmic reticulum must be quickly degraded via the ER-associated degradation (ERAD) pathway, in which the substrate is ubiquitinated and degraded by the cytosolic proteasome. N-glycans and mannose trimming reactions play an important role in glycoprotein ERAD. A specific α1-6-linked mannose residue present on N-glycans of misfolded glycoproteins was recently reported to act as a 'degradation signal' in yeast and mammalian cells. This glycan structure is recognized by lectins containing a mannose-6-phosphate receptor homology (MRH) domain. Although the ERAD pathway is known to operate in plant cells, there is very little knowledge about the glycan-dependent degradation process. By searching the *Arabidopsis thaliana* genome we discovered a yet uncharacterized MRH-domain containing protein (AtMRH1). AtMRH1 is ubiquitously expressed in different plant organs, and confocal laser scanning microscopy and Endo H sensitivity showed that it resides in the endoplasmic reticulum of plants. A putative null allele of AtMRH1 (*mrh1*) was able to prevent the degradation of the mutant brassinosteroid receptor BRI1-5, which is a well established plant ERAD substrate, and suppressed the severe growth defect of the *bri1-5* mutant. Co-immunoprecipitation experiments indicate that AtMRH1 specifically interacts with the ERAD substrate BRI1-5, but not with the wild type BRI1 protein. This suggests an important role of AtMRH1 in the plant glycoprotein ERAD pathway. Additionally, knockout mutants showed increased salt sensitivity compared to wild type plants, indicating the involvement of AtMRH1 in ER stress response reactions.

202: The human lysosomal cysteine proteinase cathepsin O is N- and O-glycosylated

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Lysosomal cysteine proteinases (cysteine cathepsins) are involved in a wide range of human diseases, including arthritis, osteoporosis and cancer. Although this family of proteolytic enzymes is generally well studied, cathepsin O has received so far only little attention. Since lysosomal trafficking of cysteine cathepsins conventionally depends on the presence of N-glycans containing mannose 6-phosphate (M6P) residues, we have now investigated the glycosylation status of human procathepsin O expressed in Sf21 and HEK293 cells. Analysis of Sf21-derived procathepsin O by ESI-Q-ToF MS revealed that both potential N-glycosylation sites (Asn62, Asn105) carry paucimannosidic N-glycans as frequently observed for recombinant glycoproteins produced in insect cells. In contrast, the N-glycan moiety of procathepsin O produced in HEK293 cells was found to consist mainly of high-mannose oligosaccharides modified with M6P residues as required for efficient sorting of soluble hydrolases to lysosomes. Mutational analysis of HEK293 procathepsin O demonstrated that both Asn62 and Asn105 are modified with M6P-containing N-glycans. Simultaneous mutation of both N-glycosylation sequons resulted in quantitative secretion of the proenzyme, indicating that the M6P recognition marker is indeed required for efficient intracellular retention and lysosomal delivery of procathepsin O. Interestingly, in silico analysis of human procathepsin O indicated a potential O-glycosylation site close to the N-terminus of the protein. ESI-Q-ToF MS analysis of procathepsin O produced in Sf21 cells demonstrated that a tryptic peptide encompassing Thr30 and Thr32 is modified with a Hex-HexNAc disaccharide, indicating the attachment of the typical insect O-glycan Galβ1-3GalNAc to one of these residues. This was further supported by lectin blots and O-glycanase digestion experiments. Subsequent studies showed that HEK293 procathepsin O is also O-glycosylated, thus establishing that cathepsin O is subjected to both N- and O-glycosylation in the course of its biosynthesis.

203: The two N-glycans of murine Cellular Repressor of E1A-stimulated Genes (CREG) are both engaged in lysosomal sorting of the protein

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Cellular Repressor of E1A-stimulated Genes (CREG) is a novel glycoprotein implicated in cellular growth and differentiation. We have previously reported that murine CREG is a bona fide lysosomal protein (Exp. Cell Res. (2008) 314: 3036-3047). Since intracellular transport of CREG to lysosomes was found to depend on its interaction with mannose 6-phosphate (M6P) receptors, we have now investigated the glycosylation status of recombinant murine CREG produced in HEK293 human embryonic kidney cells. The purified recombinant protein was found to contain M6P residues as demonstrated by M6P receptor affinoblots in combination with endoglycosidase treatment.

ESI-Q-ToF MS analysis of recombinant CREG revealed that both potential N-glycosylation sites (Asn160 and Asn216) carry Man5 to Man7 high-mannose oligosaccharides containing one or two uncovered M6P residues as required for high-affinity binding of lysosomal proteins to M6P receptors. Interestingly, the extent of M6P modification was found to be higher for glycans bound to Asn216. Site-directed mutagenesis was then used to assess the individual relevance of each N-glycosylation site for lysosomal sorting of CREG in NMuMG murine mammary epithelial cells. Intracellular retention of N160Q CREG was as efficient as observed for the wild-type protein, whereas the T218A variant was secreted to a slightly larger extent. However, the intracellular fraction of either CREG mutant with one remaining functional N-glycosylation site was quantitatively routed to lysosomes. In contrast, simultaneous mutation of both N-glycosylation sequons resulted in quantitative secretion of CREG. These results establish that the carbohydrate moiety of CREG is strictly required for lysosomal transport of the protein, and that phosphorylated N-glycans attached to either Asn160 or Asn216 are capable of mediating the interaction of CREG with M6P receptors.

204: Glycosylation enzyme sorting in the Golgi – a job for more than one tethering factor

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Vesicular sorting in the Golgi generates the characteristic differential localisation of glycosyltransferases found in Golgi cisternae. Proteins are sequentially glycosylated as they pass though the Golgi; retrograde transport is required to maintain enzyme distribution despite this forward flux. Tethering factors, including the multi-subunit COG (conserved oligomeric Golgi) complex, determine the sites of vesicle fusion and actively maintain the distribution of enzymes within the Golgi. Mutations in the COG complex cause congenital disorders of glycosylation. Here we present data describing interactions between the COG complex and additional tethering and fusion factors. Intriguingly, specific COG subunits are found to interact with different families of tethering/fusion factors, suggesting a mechanism for vesicle targeting to individual cisternae, and giving insight into COG's molecular mechanism. We have further defined these interactions using yeast two-hybrid studies and in vitro binding and go on to describe a reverse yeast two-hybrid approach to create mutants that specifically disrupt these interactions. Advancing our molecular understanding of vesicle sorting will generate new insights into the role of specific mutations in inborn glycosylation disease.

205: Changes in N-Glycosylation and Migration of A549 Human Lung Carcinoma Cells by Knockdown of Transcription Factor Sp1

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Transcription factor Sp1 is a sequence-specific DNA-binding protein, which binds the GC box in promoter and enhancer regions, and regulates the gene expression of housekeeping molecules. Sp1 has been shown to increase in a variety of cancer cells and activate the gene expression of key molecules involved in tumor growth, invasion and metastasis. Although several glycosyltransferase genes have been shown to be regulated by Sp1, effects of Sp1-knockdown on N-glycosylation and malignant phenotypes of cancer cells remain to be clarified. We established the Sp1-knockdown cells by introduction of siRNA into A549 human lung carcinoma cells. Significant decreases in L-PHA- and RCA-I-bindings were observed mainly for a 120 K protein band, which is tentatively identified to E-cadherin, in the Sp1-knockdown cells, suggesting that the N-glycosylation of E-cadherin is altered by knockdown of Sp1. When the malignant phenotypes of the Sp1-knockdown cells were examined, the anchorage-independent growth in soft agar, tumorigenic potential and migratory activity of the Sp1-knockdown cells decreased significantly when compared to the control cells. Since changes of N-glycosylation on E-cadherin affect the migration of cancer cells, the underlying mechanism for decreased cell migration was examined. It is well documented that the formation of E-cadherin/β-catenin complex results in increased cell-to-cell adhesion, and reduced cell migration. Immunoprecipitation analysis revealed that the formation of E-cadherin/ β-catenin complex increases significantly by knockdown of Sp1 with the expression levels of both molecules unchanged. Meanwhile, the tyrosine-phosphorylation of β-catenin, which is associated with cell migration, decreased in Sp1-knockdown cells when compared to the control cells. These results indicate that the altered N-glycosylation stimulates the formation of E-cadherin/ β-catenin complex, but attenuates the tyrosine-phosphorylation of β-catenin, and may lead to the decreased cell migration. To further elucidate biological significance of the altered N-glycosylation by knockdown of Sp1, how the other Sp1-regulated molecules are contributed to the decreased cell migration is under investigation.

206: Expression of MUC1 in human and murine mammary carcinoma cells change their glycosylation profile and carbohydrate-dependent adhesive properties

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MUC1 mucin is one of the most studied molecular markers associated with breast cancer. This mucin is the carrier of many O-glycans which in normal breast epithelium are synthesized as extended carbohydrate chains. However, during neoplastic transformation O-glycans undergo characteristic changes and are present as short core 1-based structures represented, among others, by T antigen (Galβ1-3GalNAc), another well established marker of brest cancer. The T antigen is synthesized by C1GalT1 which adds Gal residue to Tn antigen (GalNAc). In normal breast tissues, the core 1 T antigen is usually converted to the branched core 2 structures by the action of C2GnT, resulting in the elongation of carbohydrate chain. However, in breast cancer cells the expression of this enzyme is highly decreased which gave T antigen. This structure in breast tumors is usually cryptic and present as sialyl T antigen, because of high activity of ST3GalI. In breast cancer the major carrier of T antigen is MUC1, despite this fact there is no much known about the relations between the expression of this mucin and the occurrence of this carbohydrate antigen in breast carcinoma. Also, essentially nothing is known about the mechanisms which control the expression of C1GalT1, C2GnT and ST3GalI in normal and cancerous breast tissues. In the current studies, we present evidences that overexpression of MUC1 mucin in breast cancer cells can affect their O-glycosylation pattern by down-regulation of C2GnT and ST3GalTI. As T antigen, the product of such changes, is mostly carried by MUC1, it is the first example of the glycoprotein which directs its own glycosylation. We also show that changes in the expression of tumor-associated carbohydrate structures such as the loss of sialyl LewisX structures and appearance of T antigen could strongly affect the adhesive properties of cancer cells and in this way their metastatic properties.

207: The role of N-glycosylation on *Paracoccidioides brasiliensis* biological process and yeast-host cells interaction

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The fungus *Paracoccidioides brasiliensis* is a human pathogen that causes the paracoccidioidomycosis, the most prevalent systemic mycosis in Latin America. Here, we report the inhibition of N-linked glycosylation by tunicamycin on yeast cells of *P. brasiliensis*. In general, N-linked glycans are involved in correct protein folding, intracellular transport and prevention of proteolytic degradation. Cell wall of *P. brasiliensis* is a network of glycoproteins and polysaccharides, such as chitin, featuring several functions. The chitinolytic enzyme machinery of fungi consists of chitinases and N-acetyl-β-D-glucosaminidase (NAGase) that play important roles on the fungal cell wall metabolism. Recently, our group has described a lectin involved on *P. brasiliensis* growth/morphogenesis process, besides inducing high and persistent production of TNF-α and nitric oxide by macrophages. This lectin is endowed of NAGase activity and is supposed to be accounted for the fungal growth. We have verified herein that N-glycans are embodied on the NAGase activity from *P. brasiliensis*, consequently N-glycans are required for the adequate growth and morphogenesis of *P. brasiliensis* yeasts. In addition to the effect in the whole yeast, the induced underglycosylation has inhibited some biological activities of fungal proteins contained in crude cell extract. The results provided by this study demonstrate that N-glycosylation of *P. brasiliensis* proteins is crucial for many fungal biological processes, as well as for the yeast interaction with host cells. Financial support: FAPESP and FAEPA.

208: Purification and characterization of CD24, one of major poly-N-acetyl-lactosamine-carrying glycoproteins, in PC12 cells.

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PC12D cells, a subline of PC12 pheochromocytoma cells, extend neurites faster than PC12 cells in response to nerve growth factor (NGF) and cyclic AMP. In addition, PC12D cells differ also morphologically from PC12 cells, being flat in shape and having extended short processes without any stimulation. Previously we showed that the length and content of poly-N-acetyllactosamine (PL) chains obtained from the membrane fraction differed significantly between PC12 and PC12D, and also that NGF stimulation decreased the content of poly-N-acetyllactosamine chains of PC12 cells, but had no effect on PC12D cells. And also that one of majour poly-N-acetyllactosamine-carrying glycoproteins (PL-GP) was suspected to be CD24 molecule. Since the chemical properties of CD24 quite resemble to those of glycoprotein; it consists of a small protein core with many glycans and hydrophobic residues, we thought that CD24 could be isolated from most of cellular proteins and extracted together with glycolipids by an organic solvent that was generally used to isolate glycolipids from the cells or tissues. After the extraction by using an organic solvent, CD24 was isolated from glycolipids included the extracts by size exclusion chromatography and then purified from co-purified proteins by DEAE ion-exchange chromatography. Purified CD24 which gave a silver stained smear band on SDS-PAGE was immobilized on the nitrocellulose

membranes the same way as neoglycolipids microarray was done. Endo-β-galactosidase treatment indicated that PL chains on CD24 have some fucose residues as well as sialyl residues. But our microarray assay system did not give any binding signals of the presence of Lewis x and/or sialyl Lewis x. These results were in correspondence with those of previous experiment carried out by using less purified CD24. Thus we could confirm that the reason for the less content and having shorter poly-N-acetyllactosamine chains in PC12D cells might be originated in less expression of CD24 than PC12 cells.

209: Protein kinase C regulate morphology of microglia derived from Sandhoff disease model mice

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[Purpose] Sandhoff disease (SD) is an autosomal recessive genetic disease caused by HEXB gene mutation associated with the deficiencies of lysosomal β-hexosaminidase A (HexA) and B (HexB) as well as excessive accumulation of GM2 ganglioside (GM2) in the brain. In recent years an activated macrophage/microglia-mediated inflammatory response and morphological change have been shown to be involved in the neurodegenerative progression of GM2 gangliosidoses. Cell morphology and motility critically depend on the remodeling of the cytoskeletal architecture in response to signaling molecules. However, the signaling mechanisms involved in morphological change of the activated microglia have not been elucidated yet. In this study, to elucidate a morphological change of SD microglia, we analyzed the signaling molecules regulating morphological change of microglia cell lines derived from SD and wild-type (WT). [Materials & Methods] The microglial cell lines isolated from SD WT were characterized by immunocytochemistry. Accumulated glycoconjugates were analyzed with specific monoclonal antibodies. The expression of actin organization and the related proteins were analyzed by immunoblotting. [Results & Discussion] Actin-related proteins were significantly down-regulated in SD microglia compared with WT microglia. It was demonstrated that the microglia derived WT exhibited SD microglia-like morphology when treated with signaling inhibitors of PKC. Moreover, PKC α and β1 were significantly down-regulated in SD compared with WT. These results suggest that down-regulation of PKC alpha and beta associated with the storage of GM2 may be responsible for the aberrant morphology of SD microglia. These findings observed in SD microglia may also be crucial for the morphological change in the activated microglia. This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

210: Effects of C-mannosylated TSR-derived peptides on TGF-β signaling in kidney-derived NRK-49F fibroblasts

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C-Mannosylation is unique in that an α-mannose is directly bound to the indole C2 carbon atom of a Trp residue in proteins. Thrombospondin type 1 repeat (TSR) is a functional protein module found in thrombospondin-1 (TSP-1). TSR superfamily is a diverse group of secretory and transmembrane proteins, and includes complements, properdin, TSP-1, F-spondin, mindin, ADAMTS-like 1, and so forth. In most cases, target proteins are C-mannosylated at the first Trp of Trp-x-x-Trp in the TSR. In terms of the structural characteristics and functions of TSP-1, the Trp-x-x-Trp motif of the TSR is believed to play an important role in the binding of TSP-1 with molecules such as heparin, fibronectin, and TGF-β. We recently reported that synthesized C-mannosylated TSR-derived peptides could modulate heat shock cognate protein 70-induced signaling in the cells, suggesting that C-mannosylated TSR functions in the cell, although the mechanism is not fully understood. In this study, we examined the effect of synthesized C-mannosylated TSR-derived peptides (e.g., C-Man-Trp-Ser-Pro-Trp) on TGF-β signaling in kidney-derived fibroblast NRK-49F cells. We found that TGF-β-induced cell proliferation was suppressed by the C-mannosylated peptides, compared to the controls. In the presence of the peptides, TGF-β-induced activation of smad2 was suppressed, resulting in decrease in the expression of collagen type I. These results suggest that C-mannosylated TSR plays a functional role in the regulation of TGF-β signaling. Furthermore, we will also demonstrate our results concerning the effect of the TSR module expressed in bacteria or mammalian cells on TGF-β signaling in the cells, and discuss about possible functions of C-mannosylated TSR in the TGF-β-induced cell signaling.

211: The O-GlcNAcylation Profile of Delta-Lactoferrin, a Transcription Factor That Regulates Cell Cycle Progression and Apoptosis is Modified Durin The Cell Cycle Progression

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The GlcNAcylation is a dynamic posttranslational modification of cytoplasmic and nuclear proteins which can compete directly with phosphorylation for the same or nearby Ser/Thr residues. Alterations of the GlcNAcylation profile are observed in different pathologies and in case of breast cancer a close relationship between GlcNAcylation and tumorigenesis may exist. Down regulated in breast cancer, delta lactoferrin is a potential tumor suppressor the expression of which leads to cell cycle arrest. Delta lactoferrin is a transcription factor which controls the expression of different proteins involved in the regulation of cell cycle actors (Skp1), apoptosis induction (Bax) or mRNA turnover (DcpS). It is therefore highly regulated by posttranslational modifications. GlcNAcylation of Ser 10 inhibits its transcriptional activity while phosphorylation activates it. GlcNAcylation of Ser 10 inhibits polyubiquitination of delta lactoferrin and increases its half-live. The delta lactoferrin transcriptional complex binds to the Skp1 delta-lactoferrin response element as a phosphorylated and ubiquinated isoform. O-GlcNAc directly regulates the activities of a variety of transcription factors, suppressing or enhancing transcription, depending on the promoter and other associated proteins. Therefore we studied whether O-GlcNAc modification modifies delta-lactoferrin transcriptional activity depending of the promoter. DcpS, Bax, Fas and Rb promoters were studied compared to Skp1 promoter. Recent studies have shown that O-GlcNAc and OGT are important in cell cycle regulation. O-GlcNAc levels decrease during mitosis and increase once cells enter G1. Concomitantly, OGT mRNA levels decrease during M phase. Thus, increasing O-GlcNAc levels induce a slowing down of both S and G2/M phases whereas a reduced O-GlcNAc level impairs the G1/S checkpoint transition. Since the O-GlcNAc/P content fluctuates during the cell cycle progression, we further established the O-GlcNAc/P profile of Delta lactoferrin during the different phases of the cell cycle.

212: Effects of microRNAs on fucosyltransferase 8 expression in hepatocarcinoma cells

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MicroRNAs (miRNAs) are an abundant class of short endogenous, non coding RNA about 20-25 nucleotides in length. They can pair to a mRNA and induce its post-transcriptional repression, either by transcript destabilization, translational repression or both. Increasing evidences indicate that miRNA are involved in several physio-pathological processes, such as cell growth and differentiation, development, cancer and viral infections. Dysregulation of miRNA expression may affect known oncogenes and tumor suppressor genes, thereby having implication in carcinogenesis. It is also well recognized that aberrant glycosylation is a marker of tumoral transformation; thus it is conceivable to hypothesise that, together with other already identified mechanisms, miRNAs may also play a role in the aberrant glycosylation observed in cancer cells. To test this hypothesis, we choose as a model fucosyltransferase 8 (Fut8), to verify if miRNAs could be involved in regulating its expression in hepatocarcinoma cells. It has been reported that Fut8 activity is consistently increased in hepatocarcinoma and that this can result in increased protein core fucosylation. We have used TargetScan and PicTar tools for an initial screening of miRNA able to recognize Fut8 3'UTR. MiR-122 and miR-34a were further chosen for an experimental validation, since their downregulation during hepatocarcinogenesis is well known. HepG2 cells were transfected using miR-122 and miR-34 mimics and the effects on mRNA and protein levels were analyzed by real-time PCR and Western blot. Both miRNAs were able to decrease FUT8 protein expression at 24 and 48 hours after transfection and to affect total protein core fucosylation. The direct effect of miRNAs on FUT8 3'UTR was demonstrated using a reporter assay, in which the FUT8 3'UTR was cloned downstream the luciferase gene. These data indicate that miRNAs can be a mechanisms able to modulate expression and activity also of enzymes involved in glycoconjugate formation and that dysregulation of miRNA expression may lead to altered glycosylation of cancer cells.

213: Role of N-Glycosylation in the modulation of E-cadherin functions in cancer

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E-cadherin dysfunction is a major cause of tumor development and progression. Several mechanisms have been proposed, by our and other groups, to explain the E-cadherin dysfunction in cancer, including genetic and epigenetic alterations. Our recent studies have gathered enough evidence to propose the existence of an alternative mechanism of E-cadherin regulation in cancer, operating at the post-translational level, such as N-glycosylation. Therefore and in order to clarify the role of N-glycosylation in modulating E-cadherin's functions in cancer, we have characterized the modifications of E-cadherin N-glycans structures during the acquisition of a malignant phenotype. Furthermore, we have also addressed the

specific role of two important glycosyltransferases: N-acetylglucosaminyltransferase III (GnT-III) and N-acetylglucosaminyltransferase V (GnT-V) and their products, respectively, bisecting GlcNAc structures and β1,6 GlcNAc branched N-glycan structures, on E-cadherin expression (protein and RNA), cellular localization, biological functions and in the molecular organization of the E-cadherin/catenin complex (adherens-junctions) in a tumor context (unpublished data). In addition, we have described the existence of a functional bi-directional cross-talk between E-cadherin expression and GnT-III/GnT-V glycosyltransferases that was shown to reproduce the observations in human tumors. Altogether, our results demonstrate the importance of N-glycosylation as an alternative mechanism for the modulation and regulation of E-cadherin functionality in some carcinomas.

214: Localization of arabinogalactan-proteins (AGPs) in *Echinacea purpurea* by immunofluorescence

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Arabinogalactan-proteins (AGPs) are glycoproteins which belong to the putative active compounds of Echinacea preparations [1]. The carbohydrate moieties of AGPs from Echinacea root and herb are quite similar in composition with the main sugars 1,6-Gal*p*, 1,3-Gal*p*, 1,3,6-Gal*p* and in the side chains 1,5-Ara*f*, terminal Ara*f* and terminal GlcA*p*. Both could be classified as type II arabinogalactans [2,3]. The localization of AGPs in plant tissue of *E. purpurea* helps to understand the physiological role of AGPs and to optimize the production of pharmaceutical preparations. For immunolocalization of AGPs, the ß-glucose-Yariv-reagent (ßGlcY), binding selectively to AGPs, was used to label AGPs in sections of roots, stems and leaf stalks. After addition of antibodies against ßGlcY, raised in rabbits, secondary FITC-labeled anti-rabbit antibodies have been used for detection of AGPs in plant tissue. In roots of *E. purpurea*, AGPs are mainly located in cell walls of xylem tracheary elements, especially at the inner side of the wall and inside of pit canals [3]. Comparable to roots, the highest amounts of AGPs in stems and leaf stalks could be detected in the area of the xylem, especially in vessels and tracheids. Interestingly, the involvement of AGPs in plant vascular development has been shown before [4]. In contrast to roots, the aerial parts of the plant show additionally strong immunolabeling of sclerenchyma cells and of companion cells of the phloem. References: 1. Classen B et al. (2006) Phytomedicine 13: 688-694. 2. Classen B et al. (2000) Carbohydr Res 327: 497-504. 3. Bossy A et al. (2009) Planta Med 75: 1526–1533. 4. Motose H et al. (2004) Nature 429: 873-878.

215: The shedding of CLEC-2 is a regulated process and dependent on N-glycosylation

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C-type lectin-like receptor 2 (CLEC-2) is a newly identified type II transmembrane protein belonging to the C-type lectin family molecules, which act as a cell-surface receptor for snake venom toxin rhodocytin and tumor antigen podoplanin. We previously demonstrated that the full-length mouse CLEC-2 can be cleaved into soluble form. Elevated levels of soluble forms of membrane proteins in circulating blood in humans may reflect increased expression of membrane proteins and disease activities. In the present study, therefore, we explored the possibility that human CLEC-2 (hCLEC-2) may also be proteolytically cleaved and released as soluble forms. We have observed that the production of soluble hCLEC-2 could be induced by phorbol ester (PMA) in cells stably transfected with hCLEC-2 cDNA and also in human platelets. Further studies may explore therapeutic and diagnostic applications of soluble hCLEC-2 in platelet-related diseases. Besides, our data show that the production of soluble hCLEC-2 was prevented by treatment with tunicamycin, an inhibitor of N-glycosylation. Each single mutant the three N-glycosylation sites (N68A, N120A, N134A) and double and triple mutants confirmed that all three potential sites are N-glycosylated simultaneously. Interestingly, the proteolytic cleavage was completely prevented in the all mutants, although the single mutant was located in cell membrane. Thus, the N-glycosylation pattern constituted a control point for the modulation of the production of soluble hCLEC-2.

216: Polysaccharides as the causative agents of tension in gelatinous cell wall of plant fibers

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Fibers with gelatinous cell wall can be considered as a plant version of “muscle system”: such fibers have contractile properties, which permit to change the position of certain organs in space The best known example of tissues with contractile properties is tension wood, the development of which helps to restore the stem vertical position, if it was disturbed. Similar properties are characteristic for a wide range of other plant tissues: stems of many fiber plants (flax, hemp, ramie), coiling tendrils, twining vines, aerial roots, etc. The presence of contractile properties is determined by supramolecular structure of thick secondary wall, named gelatinous. The characteristic features of such cell wall are the absence of lignin and

xylan, which are among the major polymers of "ordinary" secondary cell wall; the longitudinal orientation of cellulose microfibrils, and the presence of pectic polymers of specific structure and properties. Cellulose of gelatinous fibers is has larger sizes of crystallite cross area in comparison with most of other plant objects. It suggests the presence of lateral interaction of cellulose microfibrils fibers, which is promoted by the same (axial) orientation of cellulose microfibril and by the absence of large amounts of lignin and matrix polysaccharides, separating the microfibrils. Tension in gelations cell wall can be achieved by the entrapping of relatively small number of matrix polysaccharides molecules between the laterally interacting cellulose microfibrils. We suggest complex rhamnogalacturonan I as such polysaccharide and describe its structural parameters necessary to fulfil such a specific role. According to our data, this polymer is able to form associates, with charged backbones on the surface and interacting side chains in the core zone. Such organization permits low affinity to cellulose and compact structure, which help to effectively separate cellulose microfibrills tending to interact laterally. Requirements for the rhamnogalacturonan I structure to make possible the formation of such associates were determined.

217: Widespread Expressions of T Cell Receptor-like Proteins in Cancer Cells

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RP215 is a monoclonal antibody generated against OC-3-VGH ovarian cancer cells and was found to recognize carbohydrate-associated epitope(s) identified preferentially in cancer cell expressed glycoproteins, known as CA215. Through MALDI-TOF MS analysis of the tryptic peptides of affinity-purified CA215, it was revealed that CA215 consists mainly of immunoglobulin superfamily (IgSF) proteins (~60%) including immunoglobulins (27%), T cell receptors (TCR) (5.7%), cell adhesion molecules (8.1%) and others. By using RT-PCR and cDNA sequencing for molecular analysis, as many as 80% of over twenty human cancer cell lines were found to express TCR or TCR-like molecules at high levels. The same conclusion was drawn with the results of Western blot assay of cancer cell lines as well as the immunohistochemical staining of human cancerous tissues. Specific antibodies against TCR as well as RP215 were found to induce apoptosis and complement-dependent cytotoxicity to cultured cancer cells. In contrast, little or no expression was observed for CD3, CD4 and CD8, all of which are known to be co-receptors or co-stimulators of functional T cells. RP215 was shown to affect gene regulations of Ig-like and TCR-like molecules expressed by cancer cells. It is hypothesized that the expression of non-functional TCR-like molecules may be relevant to immune tolerance to tumor-associated antigens by the circulating T cells. Whether the widespread expression of TCR-like proteins in cancer cells reported in this study have any implications on T cell-activated cancer immunotherapy remains to be explored. Due to the characteristic domain structures of IgSF proteins, aberrant glycosylations with unique RP215-specific epitope are likely to be detected among cancer cells. Surface bound TCR-like molecules with epitope recognized by RP215 could be one of the potential targets for RP215-based anti-cancer drugs.

218: Influence of steroid hormones on glycoconjugate distribution of canine endometrial glands in a 3D cell culture system - a lectin histochemical study

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Glycoconjugates, especially of the glycocalyx of endometrial surface epithelial and glandular cells, play an important role for gamete and embryo cognition as well as implantation during canine reproduction. Lectin histochemistry proved to be an appropriate tool to investigate cycle-dependent changes in glycoconjugate expression in the canine endometrium. The aim of the present investigation was to study the influence of steroid hormones (estrogen, progesterone) on glycoconjugate expression in a three-dimensional in vitro model of the canine endometrium. Uterine glands were isolated from four canine uteri and were cultivated together with stromal cells on BD MatrigelTM for 72 hours. Glandular structures were evaluated for differentiation and polarization during culture time in all groups by transmission electron microscopy. Hormone substitution started on day 1 (100 pg/ml ß-estradiol and 30 ng/ml progesterone, respectively), and was repeated after 24 hours. The experiment was repeated and included a control group with the standard medium. Lectin histochemistry (*Ulex europaeus* agglutin UEA I, Wheat Germ agglutinin WGA, *Helix pomatia* agglutinin HPA; concentration 10μg/ml) was performed on formalin fixed paraffin embedded samples. For comparison, the native tissue of the respective uterus and the glandular explants of the control group were analyzed.

Lectin binding patterns of the isolated glands of the control group were comparable during the whole culture period to the corresponding in vivo tissue. Of the three lectins tested, HPA served as a reliable indicator for occasional dedifferentiation of glandular structures in vitro. So far, results from progesterone treatment showed that UEA I binding to the luminal surface of the cultured glands was reduced after 48 hours which reflects the in vivo lectin binding patterns in the canine endometrium during late metoestrus.

This in vitro reconstitution of the canine endometrium works as a promising tool for experimental studies to elucidate hormonal influenced alterations of glycoconjugate expression representing the physiological changes during oestrus cycle of the native canine endometrium.

219: α2, 6-linked sialic acids on cell surface modulate the adhesion of mouse hepatoma Hca-F cells to lymph nodes

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The alterations of cell surface sialylation play a key role in the adhesion, invasion and metastasis of tumor cells. Hca-F and Hca-P cells are mouse hepatoma cell lines with high and low potential of lymphatic metastasis, respectively. Our previous study showed that N-glycosylations on CD147 are involved in the malignant phenotypes of Hca-F cells. However, the role of cell surface sialic acid in hepatoma progression remains unknown. In this study, when the two cell lines were compared for surface sialic acids by flow cytometry using SNA and MAA lectins, Hca-F cells were found to express significantly higher α2,6-linked sialic acids levels. Inhibition of the oligosaccharide expression, by treatment of the cells with neuraminidase or ST6Gal-I-siRNA significantly reduced cellular adhesion to lymph nodes in vitro and in vivo (>50%). Further, inhibition of oligosaccharides on the molecules like CD147 (one of the major carriers) caused 30–45% reduction in their adherence to lymph nodes. The inhibition also decreased their adherence by ~50% to extra-cellular-matrix components especially laminin and fibronectin but surprisingly did not altered the invasion of Hca-F cells. The cells on which the expression of these oligosaccharides was inhibited failed to attenuate the characteristic spontaneous invasion and metastasis properties of Hca-F cells. In none of the cases, however, the secretion of matrix-metallo-proteases correlated with α2,6-linked sialic acids expression. These results indicate α2,6-linked sialic acids modulate the adhesion, rather than invasion, of Hca-F cells to lymph nodes in vitro and in vivo. (This work was supported by grants from National Natural Science Foundation of China NO. 31000372, 30970648).

220: N-glycosylation is critical for the stability and insulin-mediated translocation of GLUT4 glucose transporter in HeLa cells

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Facilitative glucose transporter GLUT4 plays a key role in regulating whole body glucose homeostasis. GLUT4 dramatically changes its distribution on insulin stimulation, and insulin-resistant diabetes was often linked with compromised translocation of GLUT4 under insulin stimulation. To elucidate the functional significance of the sole N-glycan chain on GLUT4, wild-type and the glycosylation mutant GLUT4 was conjugated with EGFP and stably expressed in HeLa cells. The transfected wild-type GLUT4 was found to be functional and cell surface expression was elevated upon insulin treatment, while the glycosylation mutant lost its ability to respond to insulin. These results clearly indicate that N-glycan is critical for its insulin-mediated translocation. Moreover, we found that N-glycans contributes to the stability of GLUT4 in cells. On the other hand, kinetic parameters of wild-type and mutant GLUT4 were similar, suggesting N-glycosylation was dispensable for transporter activity. These results unveil the critical roles of N-glycan on GLUT4 glucose transporter.

Poster Session I (Monday)

Development & Differentiation

221: Biochemical characterization of AMOR, the female factor activating the pollen tube to react to the attractant LUREs

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During the fertilization of flowering plants, the pollen tube grows directionally inside the pistil and delivers two immotile male gametes to the embryo sac. Because of its difficulties with the observation, little is known about the molecular mechanism of angiosperm fertilization for more than 140 years. This pollen tube guidance has been thought to be mediated only by chemoattractants derived from target ovule. Previously we showed that in *Torenia fournieri* pollen tube attractant is secreted from the synergid cells. We have recently identified the attractants, LUREs. Using this in vitro pollen tube guidance assay, pollen tubes were required to grow through a cut style and cultured with ovules to correctly respond to the LUREs. Furthermore, a molecule, which induced competence to the pollen tube to respond to the attractant, was uncovered from ovule and named AMOR (activation molecule for response capability). AMOR was secreted from ovule, was heat-stable, and was collected in a fraction of arabinogalactan proteins (AGP) by Yariv reagent. But, it remains unknown about the nature of AMOR. To understand its role for the induction of competence, we investigated strategies to purify sufficient amount of AMOR to characterize its molecular nature, and active fractions obtained from column purification were analyzed further. It

was shown by affinity purification using lectin column that AMOR can bind some lectin (WGA, ConA, PNA). We also found that AGPs from vegetative tissues of other plant species and culture medium of some *Torenia* organs had some AMOR-like activity. Now, we are analyzing the possibility that AMOR is an AGP or a molecule associated with AGP.

222: Mass spectrometric profiling of N-glycans from *Caenorhabditis elegans* mutant embryos

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The free-living nematode *C. elegans* is a genetically and developmentally well characterized multicellular eukaryote the glycome of which has been examined by several laboratories. *C. elegans* glycans share similarities with vertebrate glycans in terms of their core structures, the most abundant N-glycans being those of the high mannose type whereas complex and hybrid N-glycans are either absent or present at low levels only. In addition, *C. elegans* contains large amounts of N-glycan structures not usually seen in vertebrates, such as paucimannose, truncated complex, highly fucosylated, O-methylated (Me), and phosphorylcholine (PC)-substituted glycans. Sialic acid residues have not been detected in *C. elegans* glycans. Since the N-glycan profile is unique for each developmental stage (L1-L4, Dauer, adult), a role for these glycans in worm development has been suggested. In our study we investigated the N-glycan profiles of *C. elegans* embryos from wild-type strain N2 and different mutants affecting development of embryos. Respective N-glycans were enzymatically released from mixed embryos populations containing one-celled embryos to pretzel stages, purified and analysed by MALDI-TOF-MS. In all embryos, high mannose glycans and the paucimannosidic species $Hex_3HexNAc_2$ were the most abundant N-glycan chains. Oligosaccharide profiles of mutant embryos were distinguished from those of wild type embryos by different relative proportions of these components and by the amount of additional further processed glycans. Thus, the synthesis of high mannose, paucimannosidic and truncated complex *N*-glycans is subtly affected in the *different* mutant embryos.

223: The Biological Roles of a putative Polypeptide GalNAc-transferase / WBSCR17

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Mucin-type O-glycosylation is one of the most common post-translational modifications of proteins, and UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferases (ppGalNAc-Ts) catalyze the initial step of the mucin-type glycan biosynthesis. We have previously identified a putative ppGalNAc-T gene, which is mainly expressed in the embryonic and adult nervous system. The gene is located in the critical region in the genome of Williams-Beuren syndrome patients, and is thus designated WBSCR17. Our in vitro assay indicated that WBSCR17 had a low but detectable ppGalNAc-T activity toward some typical mucin peptides. However, the functions of WBSCR17 in the developing and adult brain remain to be elucidated.

We investigated the in vivo roles of WBSCR17 in mammlian cell lines and zebrafish embryos. We found that a recombinant WBSCR17 protein produced in the culture cells was N-glycosylated and localized predominantly in the cis-Golgi, with a lesser amount in the ER We then identified zebrafish ppGalNAc-T orthologue genes in the zebrafish genome database, and cloned some of them including WBSCR17 from zebrafish cDNA library. The analysis by whole mount in situ hybridization indicated that WBSCR17 was expressed in the eyes and the brain in the 24 and 48 hpf embryos, while ppGalNAc-T1 was ubiquitously expressed at the same stages. When the expression of each isozyme was knocked down with morpholino antisense oligonucleotides, WBSCR17 knockdown embryos exhibited most severely altered phenotypes; malformations in the eyes and the brain, which are similar to the changes reported for radical fringe knockdown embryos. The embryos also showed an ectopic expression of some of the marker genes regulating the neuronal differentiation. These findings indicate that WBSCR17 is involved in the neuronal development of the zebrafish brain.

224: The highly glycosylated seminal plasma protein WGA16, a potential decapacitation factor, may specifically bind to sperm membrane microdomain through lectinic activity.

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As mammalian sperm reaches proximity of the egg, its surface endures a drastic reorganization, leading to capacitation. Since only capacitated sperm is able to interact with zona pellucida at the surface of oocyte, capacitation is prerequisite to fertilization. Looking for pig sperm capacitation markers, we showed that wheat germ agglutinin (WGA) epitope-bearing glycoproteins enriched in membrane microdomain decreased after capacitation. We evidenced at both pig sperm surface and

seminal plasma a highly glycosylated protein, WGA16. We observed a correlation between high sperm fertilizability and the absence of WGA16, suggesting a decapacitation function. Taking advantage of its singular affinity towards WGA lectin, we purified WGA16 from seminal plasma. Partial amino acid sequencing linked WGA16 to Zymogen Granule protein 16 homolog b (ZG16b). We confirmed that WGA16 nucleotidic sequence partly corresponds to ZG16b (79% identity). Because of its unusually high level of glycosylation, we assumed that WGA16 glycans might be crucial in the structure-to-function relationship of this potential decapacitation factor. We revealed that WGA16 exhibited a very singular glycosylation profile among seminal plasma proteins, characterized by the exclusive presence of highly N-acetylhexosaminylated structures, eventually sialylated, and by the absence of alpha-Gal residues. We hypothesize that WGA16 may specifically bind to sperm surface through lectinic recognition, especially since recent crystallographic study on human pancreatic ZG16b structure revealed a glycan binding site (Kanagawa et al., 2011). However, we cannot exclude that WGA16 itself is recognized by some specific partners at the sperm surface. Searching for binding partners, we noted the reproducible interaction of WGA16 with a 75 kDa-sperm protein, exclusively before capacitation. Identification of this protein should give new insight on how WGA16 binds to sperm surface, and how it can prevent from capacitation. Kanagawa et al., BBRC, 2011, 404, 201-5.

225: Isolation and characterization of a novel heavily glycosylated protein, WGA-gp, from pig sperm membrane microdomain

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Mammalian fertilization occurs through several steps of sperm-egg interaction. During passing through the female genital tract, sperm undergo various changes called capacitation, to gain the ability to fertilize the egg. Upon capacitation, lots of highly complex phenomena take place, including sperm surface membrane remodeling. Recently, it has been proposed that certain molecules are highly concentrated in membrane microdomains, and their reorganization during capacitaion is important in the subsequent fertilization steps. Previously we isolated microdomains from sea urchin sperm, and found several glycoproteins that are involved in such as sperm-egg adhesion and sperm motility. We especially focused on a heavily glycosylated glycoprotein, flagellasialin, in the microdomain. Several evidences showed flagellasialin regulates an intracellular Ca^{2+} that is involved in sperm motility. We wondered if this type of glycoprotein also occurred in sperm of other animals, and have searched for such glycoproteins in pig sperm. We finally detected such a polydisperse glycoprotein in the microdomain of pig sperm using wheat germ agglutinin (WGA). This glycoprotein was named WGA-gp. To understand biological function of WGA-gp, we purified the glycoprotein from pig sperm lysate, and prepared an anti-WGA-gp monoclonal antibody, which recognizes the carbohydrate part of WGA-gp. Western-blot analysis demonstrated that WGA-gp was synthesized in the epididymis, and that WGA-gp remained on the microdomain before and after capacitation. Immunofluorescent study showed that the localization of WGA-gp on the sperm surface changed depending on capacitation and acrosome reaction, suggesting that the microdomains containing WGA-gp are repeatedly rearranged on the surface during fertilization. Furthermore, anti-WGA-gp was shown to produce oscillated changes of the intracellular Ca^{2+} in pig sperm. Because intracellular Ca^{2+} is known to regulate sperm motility, WGA-gp may be involved in sperm motility. These functional features of WGA-gp resemble those of flagellasialin in sea urchin. All these results suggest that WGA-gp may regulate intracellular Ca^{2+} in collaboration with some Ca^{2+} transport proteins on the same microdomain.

226: Glycan Profile Changes Provoked by Reprogramming Transcription Factors Generating Induced Pluripotent Stem Cells

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Induced pluripotent stem (iPS) cells can be generated from somatic cells by expression of reprogramming transcription factors. It has been shown that the iPS cells and embryonic stem cells (ESCs) share lots of properties including the surface markers for the identification. They share glycan epitopes such as the stage-specific embryonic antigens (SSEA-3 & -4) and tumor-rejection antigens (TRA-1-60 & 1-81) which have been highlighted owing to their usefulness to identify undifferentiated cells with pluripotency. Even though several glycomics studies have been already carried out to elucidate the glycan profiles of ESCs, no investigation about the glycosylation patterns of iPS cells has been reported yet. In the present study, we investigated the effects of four reprogramming factors (*OCT4, SOX2, c-MYC and KLF4*) on the glycosylation patterns of human cells. After introduction of each transcription factor into human GP2-293 cells using a retroviral vector, N-glycans obtained from whole cell lysates were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Furthermore, the effects of different combinatorial introductions of transcription factors (*OCT4*/*SOX2*, *OCT4*/*SOX2*/*KLF4*, and *OCT4*/*SOX2*/*KLF4*/*c-MYC*) were investigated. Our results suggested that the portions of sialylated complex-type glycans attached with fucose were increased in the cells transfected with reprogramming transcription factor(s) containing *OCT4* or *SOX2*.

227: Characterization of two developmentally-significant *Xenopus* peptide:N-acetylgalactosaminyl-transferases

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Recent data from studies using frog embryos revealed the importance of specific polypeptide GalNAc transferases in embryogenesis, organogenesis and adult tissue homeostasis. The expression of this class of glycosyltransferases is reported to be highly regulated. Until now, the mammalian forms of these enzymes have been most characterized. Here, we would like to present the characterization two hypothetical ppGalNAc-transferase isoforms of *Xenopus laevis*, which catalyse the transfer of GalNAc to proteins, were successfully characterized. These enzymes (xGalNT-6 and xGalNT-16, respectively) are highly homologous to the human isoforms ppGalNAc-T6 (71%) and ppGalNAc-T16 (73%) and therefore similar biological functions of these enzymes in both humans and frogs can be predicted. Recombinant forms of the enzyme could be successfully expressed and secreted in *Pichia pastoris*. Testing different acceptor substrates revealed that xGalNT-6 had a broader substrate specificity than xGalNT-16; for some substrates, xGalNT-6 had the capacity to catalyze the transfer of more than one GalNAc residues. The biochemical characterization was performed by HPLC using the peptide substrates EA2 and ActR-IIB: similar kinetic characteristics, for both enzymes could were obtained for EA2, whose sequence is derived from rat submandibular gland mucin and is known to be a generic substrate for the characterization of various mammalian polypeptide GalNAc-transferases. In contrast, only xGalNT-16 showed transferase activity towards the peptide derived from the ActR-IIB receptor. In summary, the enzymatic properties of the two isoforms are similar to these reported for mammalian isoforms. However, distinct fine specificities towards peptide substrates also suggest different biological roles, which are not yet fully understood. As far as we know, this work shows also for the first time enzymatic activity of the isoform 16 from this class of glycosyltransferases.

228: β3GnT2 poly-N-acetyllactosamine (PLN) oligosaccharides are essential for proper adenylyl cyclase 3 (AC3) enzymatic function and localization in olfactory neurons

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In the mouse olfactory epithelium (OE), odorant receptor stimulation generates cAMP signals that function in both odor detection and the regulation of axon guidance molecule expression. The enzyme that synthesizes cAMP, adenylyl cyclase 3 (AC3), is coexpressed in olfactory sensory neurons (OSNs) with poly-N-acetyllactosamine (PLN) oligosaccharides by the glycosyltransferase β3GnT2. The loss of either enzyme results in similar defects in olfactory axon guidance, suggesting that glycosylation may be important for AC3 function. AC3 is extensively modified with N-linked PLN, which is essential for AC3 activity and localization. On Western blots, AC3 from the wildtype OE migrates diffusely as a heavily glycosylated 200 kDa band that interacts with the PLN-binding lectin LEA. AC3 from the $\beta3GnT2^{-/-}$ OE, however, loses these PLN modifications, migrating instead as a 140 kDa glycoprotein. Furthermore, basal and forskolin-stimulated cAMP production is reduced 80-90% in the $\beta3GnT2^{-/-}$ OE. Although AC3 traffics normally to null OSN cilia, it is absent from axon projections that aberrantly target the OB. The cAMP-dependent guidance receptor neuropilin-1 is also lost from $\beta3GnT2^{-/-}$ OSNs and axons, while semaphorin-3A ligand expression is upregulated. In addition, kirrel2, a mosaically expressed adhesion molecule that functions in axon sorting, is absent from $\beta3GnT2^{-/-}$ OB projections. These results demonstrate that PLN glycans are essential in OSNs for proper AC3 enzymatic function and localization.(Supported by NIH grant DC00953 and the Mizutani Foundation)

229: Sulfated glycan inhibits tumor cell growth by targeting BMP2/BMP receptors

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Bone morphogenetic protein 2 (BMP-2) is a member of the BMP subgroup belonging to the transforming growth factor (TGF)-beta superfamily. It binds heterodimeric receptor complexes composed of a type I receptor and a type II receptor, and transduces the related signals via the down-stream molecules, such as the P13 kinase, Smads, STATs, etc. It was clearly demonstrated that BMP-2 was implicated with angiogenesis. Heparin-like poly-saccharides posses the capacity to inhibit cancer cell

proliferation, angiogenesis, invasion, and cancer cell adhesion to vascular endothelia via adhesion receptors. Some experiments have been identified that BMP-2 interact with heparin/heparin sulfate to modulate its biological activity. The clinical applicability of the antitumor effect of such polysaccharides, however, is compromised by their anticoagulant activity. The development of define, non-toxic anion compounds may therefore provide a new strategy to interfere with angiogenic activities of heparin binding growth factors. In the experiments, we used QCM (Quartz Crystal Microbalance) to screen the potential anion polysaccharide with antiangiogenesis activities by binding to BMP2. The results showed that not all the sulfated polysaccharides can interact with BMP2. Many factors could influence the interaction, such as the linkage type, molecular size of the polysaccharide and the degree of the sulfation, etc. Interestingly, their antiangiogenesis activities were in consistent with the binding strength between sulfate glycan and BMP2. So, theoretically, we may get the ideal non-toxic anion compounds with antiangiogenesis activity by this strategy. Indeed, by QCM screening, we discovered a potential sulfated polysaccharide, which not only bind to BMP2 but also to BMP receptors to impair tumor cells growth via disrupting Smad 1/5/8 signaling (Qiu, H et al. J Biol Chem. 2010, 285, 32638-46).

230: Withdrawn

Poster Session I (Monday)

Glyco(bio)technology

231: Rhamnosidase of *Penicillium commune*

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α-L-Rhamnosidase (EC 3.2.1.40) exerting specificity towards terminal α-1,2-, α-1,4- and α-1,6-linked rhamnose residues, which are often in glycoconjugates from the environmental samples and synthetic glycosides. This property of α-L-rhamnosidase creates a possibility for its use in biotechnology for the hydrolysis of rhamnopyranosidic residues from flavonoid glycosides, such as naringin and hesperidin. This will result in higher product quality, increasing grape juice and wine natural flavors due to release of flavonoid compounds from rutinosides. In the medical practice α-L-rhamnosidase can be applied to the conversion of bioflavonoids, such as rutin and quercetin to polymers used for heart diseases prophylactics and treatment. Substances possessing antibacterial properties can be obtained from bioflavonoides, and used in cosmetology and pharmaceutical industry. There are references in a literature on α-L-rhamnosidase producers of bacterial and fungal origin. Yet, many of such a producers have a different flaws, which makes enzymes of interest unacceptable for the use in industry because of their technical or economical disadvantages. This study is dedicated to the purification, analysis of biochemical properties and substrate specisifity of two extracellular α-L- rhamnosidases (α-L-Rha-ase 1 and α-L-Rha-ase 2) from *Penicillium commune*. Two α-L-rhamnosidases from *Penicillium commune* were purified about 207 and 67-folds respectively. Activity of α-L-Rha-ase 1 was 157 U/mg protein, and 51 U/mg protein for α-L-Rha-ase 2. Molecular weight of proteins determined by gel filtration was 125 kDa for α-L-Rha-ase 1 and 105 kDa for α-L-Rha-ase 2. Both enzymes contain also carbohydrate component, represented by mannose, galactose, rhamnose and glucosamine for α-L-Rha-ase 1, galactose and rhamnose for α-L-Rha-ase 2. α-L-Rha-ase 2 was found to exhibit strong specificity towards glycon, whereas α-L-Rha-ase 1 demonstrated wide substrate specificity. Both α-L-Rha-ase 1 and 2 were inhibited by high concentrations of synthetic substrate and reaction product – L-rhamnose. Active center of both α-L-Rha-ase 1 and 2 appears to contain the carboxyl group of C-terminal aminoacid and imidazole histidine group.

232: Site-specific mutants of β-galactosidase from *Kluyveromyces lactis*

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The yeast β-galactosidase or lactase (EC 3.2.1.23) is a widely used enzyme in the dairy industry for solving lactose intolerance problems. Beta-galactosidases are widely distributed in nature, but only a few preparations are commercially available and rated GRAS. The most important sources of industrial lactases are *Kluyveromyces lactis* and *K. fragilis*, *Aspergillus niger* and *A. oryzae*. The dairy yeast *Kluyveromyces lactis* produces an intracellular lactase with a neutral pH optimum (pH 6.5-7), which is suitable for treatment of milk. In this work, β-galactosidase gene from *Kluyveromyces lactis* was cloned and expressed in *E. coli*, followed by purification and characterization of the protein. A three-dimensional model of *K. lactis* lactase was generated using the structure of *E. coli* β-galactosidase as a template. The sequence similarity between the two is 48%. On the basis of this model, several site-specific mutants were created for studying the catalytic site. The mutants have one of the following substitutions: Tyr-523 substituted with Phe, His, Cys or Asp; Glu-508 substituted with Ala or Gln; or His-416 substituted with Phe or Asn. All substitutions resulted in the loss of a large amount of the

activity, indicating an important role for the mutated residues in the β-galactosidase mechanism. Interestingly, the Y523F mutant displayed an optimal pH at 6.0-6.5, whereas the Y523H mutant had an optimal pH at 7.8-8.2. In *E. coli* β-galactosidase, the phenol group of Tyr-503 forms an H-bond with a Glu-537 oxygen. In view of this, we suggest that Tyr-523 may assist Glu-551 in its action as a nucleophile.

233: Study of synergistic activity for enzymatic chitin degradation

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Chitins have attracted particular interest for their potential conversion to N-acetylglucosamine (GlcNAc), which possesses versatile functional properties as skin moisturizers and joint-pain relievers. N,N´-diacetylchitobiose (disaccharide) also might be physiological active substance but their functions were not well studied. If we found a method to make large amount of N,N´-diacetylchitobiose easily, we would reveal their functions and are able to use them efficiently. Furthermore, N,N´-diacetylchitobiose can be used as a substrate for enzymatic synthesis of chitin oligomers which have antitumoral and immunostimulating effect.

In general, the production of GlcNAc or N,N´-diacetylchitobiose from crab shells takes a large number of processes and deleterious substances. The aim at present, is the realization of direct production of GlcNAc and N,N´-diacetylchitobiose from not only powdered chitin but also flaked chitin. To improve efficient enzymatic degradation of chitin materials, we analyzed chitinases and some secreted proteins that have synergistic effects for degradation from *Streptomyces griseus* which enzymes use for foods.

Major chitinases which mainly produce N,N´-diacetylchitobiose from chitin polymers were determined from the culture supernatant of *S. griseus* with flaked chitin as the sole carbon source. We isolated major genes that were expressed under chitin inducible conditions. We isolated total RNA from the same chitin treated culture and used it as the template for real time PCR. Three chitinases (ChiII, III and C), one of the serine proteases which have a chitin binding domain-like sequences and all four putative chitin binding proteins showed increment of mRNA accumulation by flaked chitin treatment.

Then isolated chitin inducible genes were introduced in *Eschericha coli* to obtain recombinant proteins for enzymatic analysis. Recombinant proteins of ChiII, III, a serine protease and one of chitin binding protein genes were expressed and purified. Studies for their synergistic effect will be discussed.

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234: Lipopolysaccharides of *Rahnella aquatilis*

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From over 100 species of microorganisms identified before 1995 as representatives of Enterobactereaceae family only 20 draw attention of microbiologists whereas the others, including *R. aquatilis*, remained practically unexplored. Exept of water reservoirs, *R. aquatilis* were also obtained from soil (mostly rhizosphere of cereals: corn, wheat, rice) and clinical material. *R. aquatilis* is a heterogeneous on biological and biochemical properties species, which systematics have many unsettled questions. It is known that composition and structure of lipopolysaccharide (LPS) is one of the recognized chemotaxonomic criteria. The lipopolysaccharides of *Rahnella aquatilis* were isolated from bacterial cells by phenol-water extraction. The structural components were obtained by mild acid degradation of the LPS followed by GPC on Sephadex G-50. The predominant monosaccharides of core oligosaccharides were glucose, galactose. The presence only one high-molecular fraction O-PS was characteristic feature of all *investigated* strains. The predominant monosaccharides of O-PS tested were galactose, glucose, mannose, rhamnose and fucose. 3-Oxytetradecanoic (48,9-93,1 %), dodecanoic (3,2-12,1 %), tetradecanoic (4,1-25,3 %) and hexadecanoic (2,8-15,3 %) acids have been obtained in lipids A of LPS tested. The presence in lipid A of *R. aquatilis* only 3-oxytetradecanoic acid which is characteristic for Enterobacteriaceae proves the correct applying of isolated strains to this family. By double immunodiffusion in agar it was shown, that *R. aquatilis* LPS displayed antigenic activity in homological systems. The results of serological cross reactions indicate the immunochemical heterogeneity of *R. aquatilis* species. The results of the comparative toxicity studies of native and modified complexes of *R. aquatilis* 96U037 LPS indicates that, due to the modification of LPS complex tin with benzoilgidrazon-4-dimetilaminobenzaldehyde observed toxicity and leads to the disappearance of pyrogenic effect. All scored derivatives completely lost antigenic activity in both homologous and heterologous systems in, which may indicate that the interaction of modifying complexes with certain groups that comprise the epitope.

235: Novel binding specificities of *Ricinus communis* agglutinin (RCA120) and *Erythrina cristagalli* lectin (ECL)

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Lectins such as RCA_{120} and ECL are used extensively as research tools to detect and target oligosaccharides containing terminal β-D-galactose. Here we use mini-carbohydrate microarray to investigate different sulfated galactan oligosaccharide derivates' binding to RCA_{120} and ECL. The interactions of oligosaccharides with RCA_{120} were further confirmed by an inhibition assay on the microtiter plate. It was demonstrated that both RCA_{120} and ECL displayed a higher affinity for structures with terminal Galβ1-4 rather than Galβ1-3 linked oligosaccharides by binding assay as previously documented. However, it has not been shown that an anhyro-form of galactose (anGal) can be accommodated by RCA_{120}. More interestingly, RCA_{120} binding to Galb1-4 can be enhanced by sulfation at the 2-O-position or 6-O-position of the Gal, but the binding was completely abolished by sulfation at the 4-O-position of the Gal. Inhibition assay on the microtiter plate further confirmed the conclusion above. In contrast, ECL only recognized intact structure sequence of Galβ1-4GlcNAc patterns, did not accommodate any sulfated Galβ1-4 sequences or anhyro-forms of galactose.

236: A robotics platform for automated high-throughput glycan analysis of the serum glycome

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Aberrant glycosylation plays a significant role in disease development and manifestation. The potential of glyco-biomarkers in the diagnosis and monitoring of disease is now being realised and offers a novel and innovative approach to the discovery of clinical markers. To discover and validate novel glycan biomarkers, large sample sets from different disease stages require comprehensive glycan analysis. To that end we have developed, optimized and automated a 96 well plate glycan preparation strategy. A robotic platform has been adapted which facilitates high-throughput sample preparation. Serum samples are immobilised prior to reduction and alkylation which has significantly improved sample recovery. Released glycans are then labeled with 2-AB in an integrated incubator. Rapid removal of excess fluorescently labeled material is accomplished by employing a 96 well solid phase extraction (SPE) plate. This technology offers the opportunity to prepare and analyse glycans from a large cohort of samples facilitating not only biomarker discovery and validation but also offers the opportunity to monitor the glycosylation profile during biotherapeutic production in the biopharmaceutical industry. In addition to this, combining this high-throughput glycomics technology with genome wide association studies (GWAS) permits a more rapid investigation into the genetic regulation and biological roles of glycan structures.

237: Development of a multiple enzyme-processing method and high-resolution HILIC UPLC glycoprofiling of a CHO cell-expressed complex glycoprotein.

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Changes in the glycosylation status of glycoproteins often can influence their bioactivity, efficacy and clearance. Monitoring of such changes during the technical development of recombinant therapeutic glycoproteins is often necessary to ensure maintenance of desired quality attributes and to assess process consistency. Development of facile glycomonitoring methods can be a challenging task, especially in the case of non-IgG therapeutic proteins with complex glycosylation profiles. We describe here the development of a simplified approach used for monitoring of potential changes in N-glycosylation profiles of a Chinese hamster ovary cell-expressed recombinant protein, carrying a complex mixture of neutral and highly negatively charged (phosphorylated and sialylated) glycans. We applied this approach as part of an assessment performed during the development of a "working cell bank." N-glycans were processed by a facile spin column format with a 0.2μm hydrophobic PVDF membrane. This was proven to be a viable and user-friendly approach for either the release of N-glycans alone with PNGase F enzyme, or the more complex sequential removal of negative charge with Sialidase A and alkaline phosphatase, followed by the release of the charge-neutralized glycans by PNGase F. A high-resolution HILIC UPLC glycoprofiling method was developed for the separation of fluorescently labeled (2-aminobenzoic acid) glycans, and the advantages of this method are compared to standard HILIC LC separation. Orbitrap UPLC LC-MS analysis in a negative ion mode was developed and used to characterize obtained glycan profiles. Subsequently, compositional assignment was performed for a majority of individual glycan peaks from the acquired accurate masses. Finally, using this approach, the glycans present on the material from a "working cell bank" were successfully assessed and examples of data are presented and discussed.

238: Towards biosimilar monoclonal antibodies: Batch-to-batch profiling using mass spectrometry and N-glycan mapping

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Biosimilars are defined as biological medicinal products comparable (but not identical) to their reference market products in quality, safety and bioefficacy. Monoclonal antibodies (mAbs) are subject to complex post-translational modifications which are sensitive to subtle changes in manufacturing processes, e.g. N-glycosylation, N- and C-terminal protein chain heterogeneity (pyroglutamate formation, lysine clipping), and therefore require detailed and reliable analytical control testing. In the present work we show that by using ESI-TOF-MS analysis of the intact mAbs or their light and heavy chains, exact mass determination of variants due to micro-heterogeneity of glycosylation and N- and C-terminal modifications or truncations can be assessed. Using this mass spectrometry platform, we are able to monitor batch-to-batch consistency of different therapeutic mAbs in a highly reliable and straightforward manner.

We show that Herceptin®, a therapeutic mAb that is widely used in breast cancer treatment, exhibits batch-to-batch variations with respect to N-glycosylation, which is known to effect ADCC, CDC and Fc-gamma receptor binding. No difference was observed in the C-terminal lysine clipping. Our highly sensitive ESI-TOF-MS analysis in combination with complementary analytical HPAEC-PAD mapping enabled us to identify batches with variations in galactose content and to identify additional high-mannose type structures. All Herceptin® batches contained invariantly about 15% of N-glycan structures lacking proximal fucose (including 1-4% of oligomannosidic structures).

Different batches of the mAb market products Remicade® and MabThera® and biosimilar mAb preparations produced from CHO cell clones were found to differ in their degree of C-terminal lysine clipping as well as N-terminal pyroglutamate content.

Taken together, our MS platform in combination with HPAEC-PAD enables straightforward and highly effective assessment of mAb micro-heterogeneities and assessment of safety and bioefficacy of biosimilar preparations and supports the development of biosimilar mAbs from early research to the final batch-to-batch consistency control.

239: Chemoenzymatic Synthesis of Tetraantennary Bisected Core Fucosylated N-glycan Derivatives: printed on the CFG glycan microarray

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A tetraantennary, bisected, core-fucosylated N-glycan with terminal N-acetylglucosamine (GlcNAc) was chemically synthesized as reported by Eller et al. (2). The reducing end of the N-glycan was conjugated by a 2-aminomethyl N,O-hydroxyethyl spacer for immobilization on the NHS-activated array slides (3). After purification by a graphitized carbon column to remove the excess amounts of unbound spacer, the N-glycan was systematically elaborated with following enzymes. Each enzymatic step was followed by purification on carbograph or Sephadex G-25 to separate products from the reactants and enzyme. Production of the desired compound was confirmed by matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS). The first elongation step was carried out by bovine β1-4 galactosyltransferase with UDP-Gal to make terminal lactosamine (LN) on the tetraantennary N-glycan. MS analysis confirmed the galactose incorporation on all four branches, where the bisecting GlcNAc remained intact. Further elongation with rat α2-3 sialyltransferase and CMP-Neu5Ac generated terminal sialyllactosamine on all branches. Complete sialylation was followed by enzymatic reaction with β1-4 N-acetylgalactosyltransferase from *Campylobacter jejuni* to make the Sd^a antigen. Generation of Sd^a terminal motifs on four branches further confirmed the successful stepwise elaborations that provided substrate for each subsequent enzyme. Compounds were test printed on the NHS-hydrogel slides and detected by several relevant lectins. The results show another example of the marriage of chemical and enzymatic methods to achieve the synthesis of complex N-glycans. Inclusion of these bisected, core fucosylated multiantennary glycans on the diverse CFG glycan array will enhance the potential of the array to detect specific ligands in glycan-protein interaction studies.

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240: Strict binding specificity for core (α 1-6) fucosylated N-glycans and biological activity of small-sized lectins from the red alga *Hypnea japonica*

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Three isolectins (hypnins A1-A3) from *Hypnea japonica* are polypeptides composed of 90 amino acids including two intrachain disulfide bonds and belong to a new lectin family. They have favorable properties such as a small size (9 kDa), a monomeric form, and extreme thermostability for applications. They inhibit the platelet aggregation induced by either ADP or collagen. However, their carbohydrate-binding properties were unknown except that they had no affinity for monosaccharides. Here we report their carbohydrate-binding specificities and biological activities as well as the hypninA2 cDNA cloning and the preparation of rhypninA2.

In frontal affinity chromatography with about 100 pyridylaminated oligosaccharides, the isolectins commonly bound only core (α 1-6) fucosylated N-glycans, and did not the other (α1-2), (α 1-3), and (α 1-4) fucosylated glycans. The binding affinity remarkably increased by the addition of bisecting GlcNAc to the N-glycans. The novelty of the carbohydrate-recognition mode of hypninA was confirmed by surface plasmon resonance analyses on an immobilized glycoprotein with and without core (α 1-6) fucose. Such specificity of hypninA is clearly distinct from those of other known fucose-binding lectins assayed as references, making it a valuable tool for cancer diagonosis and quality control of antibody medicines. They were also confirmed to have both anti-coagulant and anti-angiogenesis activities at lower concentrations, as predicted from the existence of the sequence motifs similar to the active sites of an adhesive protein (fibrinogen) and an anti-coagulant peptide (hirudin) for the former activity.

The full-length cDNA cloning of hypninA2 reveals that hypninA2 cDNA encodes 17 residues of signal peptide, 39 residues of propeptide and 90 residues of mature polypeptide. A recombinant hypninA2 (rhypninA2) was generated in *Escherichia coli* with pET system using the *E. coli* codon-optimized construct and 9.0 mg of active rhypninA2 was yielded from 1 L of cell cultures. The recombinant should contribute to structural analysis and applications of the lectin.

241: The cherry on the cake: Expression of sialylated human Erythropoietin in plants

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Recombinant human erythropoietin (rhEPO) is one of the leading biopharmaceutical products on the market. In mammals this glyco-hormone is decorated with branched and sialylated N-glycans which are major determinants in the efficacy of the drug. Thus the production of rhEPO is currently restricted to mammalian cell expression systems. As higher eukaryotes, plants have the advantage that they carry out complex glycosylation, but they do not generate branched or sialylated N-glycans. In previous studies we showed that N. benthamiana, a tobacco related plant species widely used for recombinant protein production, is well suited for the modification of the N-glycosylation pathway towards human type glycosylation, including terminal sialylation (Strasser et al., 2008, Castilho et al., 2010). In this study we set out to generate rhEPO carrying a human type glycosylation pattern using plants as expression host. In a first set of experiments we co-expressed the mammalian enzymes responsible for the generation of branched structures (i.e. N-acetylglucosaminyltransferase III, IV and V) along with hEPO in N. benthamiana. As a result rhEPO was homogeneously decorated with bisected N-glycans and with tri- and tetraantennary structures (Castilho et al., 2011). In another set of experiments, the transiently co-expression of six human genes involved in the sialic acid pathway (Castilho et al., 2010) resulted in the production of hEPO with biantennary sialylated structures. Our results towards glycoengineering of rhEPO demonstrate the great potential of plants for the generation of proteins with a tailor made N-glycosylation profile. Moreover by combining branching and sialylation we expect to generate in the near future plant made rhEPO with multiantennary sialylated structures which are crucial for an enhanced in vivo activity.

242: Generation of glyco-engineered rice *(Oryza sativa)* for the production of recombinant glycoproteins lacking plant-specific immunogenic N-glycans

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The rice α-amylase 3D promoter system, which is activated under sucrose-starvation conditions, has emerged as a useful system for producing recombinant proteins. However, using rice as production system for therapeutic proteins requires modification of their N-glycosylation pattern, because of the immunogenicity of plant-specific sugar residues. In this study, glyco-engineered rice were generated as a production host for therapeutic glycoproteins, using RNA interference (RNAi) technology to down-regulate the endogenous α1,3-fucosyltransferase (α1,3-FucT) and β1,2-xylosyltransferase (β1,2-XylT) genes. N-linked glycans from the RNAi lines were identified, and their structures compared with those isolated from wild

type suspension cells. The Δ3FT/XT-9 glyco-engineered line with significantly reduced core α1,3-fucosylated and/or β1,2-xylosylated glycan structures was established. Moreover, level of the plant-specific α1,3-fucose and/or β1,2-xylose residues of recombinant human granulocyte-macrophage colony stimulating factor (hGM-CSF) produced from N44+Δ3FT/XT-4 glyco-engineered line co-expressing ihpRNA of Δ3FT/XT and hGM-CSF was significantly decreased compared to N44-08 transgenic line expressing hGM-CSF which had been reported previously. None of the glyco-engineered lines differed from the wild type in cell division, proliferation or ability to secrete proteins into the culture medium. (This Study was supported by Technology Development Program (609004-05-1-SB130) for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea, and also by a grant from the Next Generation New Technology Development Project (10030122) of the Ministry of Knowledge Economy of the Republic of Korea).

243: Functional Characterization of a Peptide:N-Glycanase identified from Yeast *Yarrowia lipolytica*

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Several classes of peptide: N-glycanse (PNGase) enzymes have been identified and used for release of N-glycans from glycoproteins. Specifically, for deglycosylation of pant and insect glycoproteins, PNGase A purified from almonds has been employed because most widely used enzyme, PNGase F, cannot cleave N-glycans containing the fucose α(1,3)-linked to the innermost N-acetylglucosamine. In this study, we identified a new PNGase enzyme from *Yarrowia lipolytica*, of which amino acid sequence shows weak homology with PNGase A. This gene was cloned and successfully expressed in *Pichia pastoris* using the commercially available vector for secretory expression. The prepared enzyme, designated as PNGase Y, was biochemically characterized for optimum ranges of pH and temperature, enzyme kinetics and substrate specificity. It was shown to have a broad substrate specificity cleaving various types of N-glycans including high-mannose and complex type glycans. Especially, it could release the glycans containing core α(1,3)-fucose found in plant and insect as well as bi-phosphorylated glycans for lysosomal proteins, which are not good substrates for PNGase F. Moreover, PNGase Y was shown to release N-glycans efficiently from denatured glycoprotein as well as glycopeptide. It is a great advantage compared to PNGas A which requires an additional step of protease digestion due to the preference for glycopeptides as a substrate. Taken together, we suggest that PNGase Y will be a good tool to release various types of N-glycans with broad range of substrate specificity under favorable conditions without the need of glycopeptides preparation.

244: Fluorescent Probes of *Aspergillus oryzae* Lectin Fused with Fluorescent Proteins Are Internalized into Intracellular Compartments via Endocytosis

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Aspergillus oryzae lectin (AOL) encoded by the *fleA* gene has been reported to bind to fucosylated glycans. In our previous study, the *fleA* gene was fused with genes of fluorescent proteins to generate fluorescent probes for detecting fucosylated glycans. The binding capabilities of the resulting constructs were confirmed by lectin blot and cytochemical staining. Here, we describe the unexpected internalization properties of the FleA-fluorescent fusion proteins. During cell imaging experiments, we surprisingly found that these proteins could be internalized into intracellular compartments when applied to live cells. In contrast, their fluorescences were observed only on the cell surface when applied to the fixed cells or the metabolically arrested cells at low temperature. These results suggested that FleA-fluorescent fusion proteins first bound to the fucosylated glycoproteins located on the cell surface and then could be internalized by the active cellular process. Competition experiments using a large amount of free fucose indicated that these binding and internalization were specific to fucosylated glycans. Moreover, the internalization process was shown to occur depending on a clathrin-mediated pathway by endocytosis inhibitor assay. Taken together, FleA-fluorescent fusion proteins can be employed as a vesicle for delivery of useful materials to the inside of cells as well as a valuable fluorescent probe for detection of fucosylated glycans.

245: In silico analysis of Completely Sequenced Microbial Genomes Reveals the Ubiquity of N-linked Glycosylation in Prokaryotes

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Glycosylation of proteins in prokaryotes is known since the last few decades. Glycosylated proteins are diverse in their function which is evident by examples like chaperonin, some enzymes, pilin, flagellin, and the S-layer glycoprotein. Even in terms of localization diversity is reflected as these proteins are not confined at one place in cell rather they can be found inside the cell, on cell surface

as well as in the periplasm. Glycosylation has been shown to be associated with a wide range of biological phenomena. Characterization of the various types of glycans and the glycosylation machinery are critical to understand such processes. However, glycan structures and/or the glycosylation pathways have been experimentally characterized in only a small number of prokaryotes. Even this has become possible only during the last decade or so, primarily due to technological and methodological developments. In view of this, the experimentally characterized pgl system of *Campylobacter jejuni*, responsible for N-linked glycosylation, has been used in this comparative genomics study to identify their homologs in 865 prokaryotes whose genomes have been completely sequenced. This study shows that only a small number of organisms have homologs for all the pgl enzymes, and a few others have homologs for none of the pgl enzymes. Most of the organisms have homologs for only a subset of the pgl enzymes. There is no specific pattern for the presence / absence of pgl homolog vis-á -vis 16S rRNA sequence-based phylogenetic tree. This may be due to differences in the glycan structures, high sequence divergence among the members of different pgl enzyme families, phenomena of horizontal gene transfer and non-orthologous gene displacement. Homologs of pgl enzymes are found in organisms belonging to all the groups considered in the study i.e., archaea, firmicutes, proteobacteria (alpha, beta, gamma, delta and epsilon), etc. The presence of homologs for pgl enzymes is not correlated with either their habitat, pathogenicity, energy generation mechanism, etc. This hints towards the ubiquity of N-linked glycosylation in prokaryotes. This study facilitates the experimental characterization of these genes and in the identification of novel targets for designing drugs, diagnostics, and engineering of therapeutic proteins.

246: Single-Molecule Force Spectroscopy for Studying Kinetics of Enzymatic Elongations of glycoconjugate

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In this study, we report the observation and dynamic kinetic analysis of dextran elongation, which is catalyzed by dextransucrase (DSase), by single-molecule force spectroscopy. DSase is a glucosyl transferase that catalyzes the transfer of a D-glucose unit in sucrose to a D-glucose at the non-reducing end of a dextran acceptor. We directly observed the elongation process from the shifts of rupture peaks between the isomaltoheptaose (dextran 7-mer)-immobilized probe and the DSase-immobilized mica surface in the presence of sucrose as a monomer. We could kinetically achieve a single-molecule enzyme reaction as a dextran elongation reaction using the distance shift of the rupture peak between the dextran oligomer-immobilized probe and the DSase-immobilized mica surface. This technique can also be applied to enzyme reactions on cell surfaces. We also examine the elongation of chondroitin by chondroitin polymerase, by single-molecule force spectroscopy and high-speed AFM. We could observe the elongation of individual sugar chain on the singleenzyme-immobilized mica surface.

247: Novel anti-HIV-1 mechanism of pseudo-proteoglycan, conjugate of unsulfated glycans with poly-L-lysine, is different from that of sulfated polysaccharides

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A proteoglycan (PG) consists of a core protein combined with more than one glycosaminoglycan chain. We synthesized unique pseudoproteoglycans (pseudoPGs) that simulate PG macromolecular structures with combinations of different sizes and different types of glycans and core polymers to elucidate the biological activities of pseudoPGs. A novel pseudoPG synthesized using 10 kDa poly-L-lysine (PLL) with 10 kDa dextran (Dex) by reductive amination exhibited remarkable anti-HIV-1 activity, although neither PLL nor Dex has such activity. The pseudoPG showed IC_{50} 16–80 times lower than that of sulfated dextran or heparin against BaL and equal to that of sulfated dextran or heparin against IIIB virus, indicating that PLL-Dex was markedly more effective against R5 virus than sulfated polysaccharides. PLL-Dex at 100 μg/ml significantly inhibited IIIB virus adsorption to human glioma NP-2 cells expressing the HIV-1 coreceptor CXCR4, and subsequent virus entry into the cells was almost completely prevented; heparin at the same concentration reduced the virus only by half, indicating that PLL-Dex inhibits both virus adsorption and cell invasion processes to prevent infection. To examine whether PLL-Dex binds to HIV-1 or cells, the reactivities with smear preparations of un- or IIIB-infected MOLT-4 T-lymphocytes were detected using a monoclonal antibody. PLL-Dex was shown to bind to viruses budding on infected MOLT-4 cells but not to uninfected cells. When lysates of MOLT-4 were separated by SDS-PAGE and transferred to a PVDF membrane, several protein bands were observed to bind to PLL-Dex, indicating that PLL-Dex binds to intracellular proteins. Membrane analysis using separated HIV-1 proteins demonstrated that PLL-Dex bound to viral core proteins and endonucleases but not to envelope glycoproteins including GP160. Flow cytometry demonstrated that Dex-PLL bound to MOLT-4-IIIB cells at a site different from that of heparin. This study proposes the use of the neoglycoconjugate, pseudoPG, to develop a novel anti-HIV-1 treatment.

248: Sulfated polysaccharides of *Delesseria sanguinea:* Pharmacological activity and quality in dependence on the isolation procedure

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The red seaweed *Delesseria sanguinea* (Hudson) Lamouroux (D.s.) dominantly populates a large artificial reef in the Baltic Sea at Nienhagen near Rostock, Germany. It contains substantial amounts of sulfated polysaccharides (D.s.-SP), which consist of a homogenous fraction of branched sulfated xylogalactans (gal:xyl ~ 5.4) and exhibit a pharmacological profile indicating anti-inflammatory and anti-skin aging potencies. Compared with heparin, D.s.-SP revealed stronger inhibitory effects on the enzymes elastase, hyaluronidase, heparanase, collagenase (MMP-1) as well as on alternative and classical complement activation, cell adhesion to P-selectin and cytokine release from LPS-activated monocytes, but have only moderate anticoagulant activity. Their hyaluronidase and complement inhibitory activities proved even superior than those of the anti-inflammatory ß-1,3-glucan sulfate PS3. Crucial for an economic use is the availability of adequate amounts of D.s.-SP with reproducible high quality. For evaluation and optimization, 30 D.s. batches were harvested and extracted since 2005 resulting in almost 200 D.s.-SP batches, which were analyzed and tested. By a standardized procedure (extraction (EX) with water for 8h at 85°C), the D.s.-SP can be isolated in reproducible high quality. Only the yields season-dependently varied from 6.1%-17.9% (mean 11.9%). However, as found by a second 8h-EX, the D.s.-SP are not completely extracted after the first 8h-EX. Subsequently modified EX-procedures led to following yields: 8.8% (1x8h-EX), 13.3% (2x4h-EX), 15.0% (2x2h-EX) and 17.9% (4x2h-EX). Consequently, a 2x2h-EX (15.0%) seems to be a rational compromise. Moreover, the D.s.-SP, obtained by shorter EX, contained less glucose, which partly represents co-extracted starch: 14.4% (1x8h-EX), 10.92% (2x4h-EX), 9.0% (2x2h-EX) and 11.74% (4x2h-EX). The glucose content was further reduced by precipitating the extracted D.s.-SP with 70% instead of 90% (v/v) ethanol. In conclusion, after stepwise optimization of the isolation procedure, the D.s.-SP from Nienhagen are ready for an economic use. Acknowledgements: This project is financed by the EU (FIAF/EFF) and the LFALF Mecklenburg-Vorpommern.

249: Characterization and quantification of the N-glycosylation of marketed recombinant therapeutic glycoprotein products

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Most of biopharmaceuticals are glycosylated proteins; the direct effect of N-glycans on quality, safety and therapeutic efficacy of the biopharmaceutical products emphasize the significance of detailed glycan analysis during pharmaceutical development and manufacturing processes.

We have established a platform involving complementary analytical methods for glycan analysis by using highly sophisticated chromatographic approaches under cGMP regulations (high resolution HPAEC-PAD analysis on CarboPac PA200 columns of N-glycans without requiring prior derivatisation; normal phase HPLC analysis of fluorescent labeled glycans) and state-of-the-art mass spectrometric analysis (MALDI-TOF MS, ESI-TOF MS/MS-MS).

In the current study the N-glycosylation profiles of recombinant biopharmaceutical market products from different manufacturers (originator products, me-too and biosimilar products, e.g.: erythropoietin, follitropin-ß, IFN-ß, coagulation Factor IX and tissue plasminogen activator) were analysed and quantified using analytical methods as described above with respect to sialylation (including the presence of O-acetylated sialic acids) and antennarity status, branching, NeuGc content, Galα1-3Gal-R content, presence of oligomannosidic glycans (neutral and phosphorylated), terminal galactosylation, Lewis-type fucosylation and presence of sulphated glycans. Among the different biopharmaceutical glycoprotein market products analyzed, the N-glycans from e.g. four different EPO market products (Eporatio®, biosimilar Epoetin alfa HEXAL® , Erypo® and NeoRecormon®) where found to predominantly comprise fully sialylated tri- and tetraantennary N-glycans. Comparison revealed significant differences in the sialylation status (bearing tetrasialylated N-glycans in proportions between <60% and >70%) and the content of phosphorylated oligomannosidic glycans (which were found to be present in up to 9% of the EPO protein products). In some of the EPO products up to 25% of the sialic acid residues were found to be O-acetylated (with 1 or 2 O-acetyl-groups).

250: Biochemical characterization of Csav-ST3Gal I/II, a sialyltransferase from the sea squirt *Ciona savignyi*

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Sialyltransferases are a group of the glycosyltransferase transferring sailic acid from CMP-Neu5Ac to the non-reducing terminal position of oligosaccharides attached to glycoproteins and glycolipids. Animal sialyltransferases are grouped into four families according to the linkage specificity of their reaction: ST3Gal I-VI, ST6Gal I-II, ST6GalNAc I-IV, and ST8Sia I-VI. The catalytic domains of sialyltransferases contain four highly conserved regions designated as the sialylmotifs- L (Large), S (Small), motif III, and motif VS (Very small). These well conserved sialylmotifs suggested that all sialyltransferases had been evolved from a common ancestor. At least 80 sialyltransferases have been known in vertebrate linage, however, only a few have been characterized in non-vertebrates. In this study, an invertebrate sialyltransferase (Csav-ST3Gal I/II) identified from the sea squirt *Ciona savignyi* was functionally characterized by using recombinant enzyme expressed in *Saccharomyces cerevisiae*. Csav-ST3Gal I/II is localized to Golgi membrane when expressed in yeast. Enzymatic assays indicate that Csav-ST3Gal I/II is capable of transferring sialic acids to the oligosaccharides attached to asialofetuin. Substrate specificities and kinetic properties indicated that Csav-ST3Gal I/II prefers O-glycans rather than N-glycan of asialoglycoproteins as substrates but it does not accept glycolipid as substrates. Further, it has been shown that Csav-ST3Gal I/II catalyzes the formation of α(2,3)-linkage by a lectin blot analysis with *Maackia amurensis* lectin and by the linkage-specific sialidase treatments. The Csav-ST3Gal I/II has three putative N-glycosylation sites in its stem region and N-glycosylation at these sites might important for the proper folding and trafficking of the enzyme in vivo. Interestingly, one glycosylation site of Csav-ST3Gal I/II was occupied with N-glycan when expressed in yeast. However, this glycan seems not to be essential for in vitro catalytic activity since its removal by peptide N-glycosidase treatments has no effect on catalytic activity.

251: Primary structure, carbohydrate-binding specificity and antiviral activity of the novel lectin from the green alga *Boodlea coacta*

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High mannose-binding lectins are found from various taxonomy. Some of these lectins exhibit a strong antiviral activity against HIV, influenza virus, and other viruses. A novel lectin BCA from the green alga *Boodlea coacta* was efficiently obtained using the yeast mannan-Cellulofine affinity column. Detailed oligosaccharide-binding specificity of BCA by a centrifugal ultrafiltration-HPLC assay using 42 pyridylaminated oligosaccharides revealed that BCA bound to high mannose-type N-glycans but not to complex type, hybrid type, core structure of N-glycans or oligosaccharides from glycolipids, and that the BCA preferentially recognizes the nonreducing terminal α1-2 mannose cluster as a primary target. As predicted from carbohydrate-binding propensity, this lectin inhibited the HIV-1 entry into the host cells at an EC_{50} of 8.2 nM. High affinity binding ($K_A = 3.71 \times 10^8$ M^{-1}) of BCA to the HIV envelope glycoprotein gp120 was demonstrated by surface plasmon resonance analysis. Moreover, BCA showed the potent anti-influenza activity by directly binding to viral envelope hemagglutinin against various strains including a clinical isolate of pandemic H1N1-2009 virus. The full-length sequence of BCA by protein sequencing and cDNA cloning revealed that BCA consisted of a signal peptide, a C-terminal propeptide and 3 internal tandem-repeated domains. BCA contains the sequence motifs similar to the carbohydrate-binding site of *Galanthus nivalis* agglutinin (GNA), which also consists of a signal peptide, a C-terminal propeptide and three subdomains, despite having no overall sequence similarity. Recently we cloned two BCA cDNA isoforms encoding BCA1 and BCA2, showing 90% amino acid sequence identity between each other. Using *Escherichia coli* expression system, we generated recombinants using different synthetic constructs encoding BCAs with the C-terminal propeptide (pro-rBCAs) or without (rBCAs). Interestingly, rBCAs represented hemagglutination activities, whereas pro-rBCAs did not. It suggests that the C-terminal propeptide blocks the carbohydrate-binding activity of BCAs as well as GNA. Here we report that BCA is the first HIV- and influenza-virus–inhibiting protein from the green algae.

252: Solubility enhancement of insoluble drugs by the complexation with sinorhizobial oligosaccharides

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Sinorhizobial oligosaccharides, cyclosophoraose (Cyclic b-(1,2)-glucan, Cys) or succinoglycan dimer has unique structures and high solubility which make it potent for solubilizer in host-guest inclusion complexation. Cyclosophoraose(Cys) isolated from *Rhizobium leguminosarum biovar trifolii* TA-1 is unbranched cyclic β-(1,2)-glucan which enhanced the solubility of insoluble drugs such as salicylic acid and naproxane. Succinoglycan dimer originated from *Rhizobium meliloti* is a linear oligosaccharide which has specific substituent of two acetyl and pyruvyl groups and one to four succinyl groups, enhanced the solubility of a highly hydrophobic drug haloperidol. In this study, sinorhizobial

oligosacchiardes and poor water soluble drugs' complexation much increased their solubility compared to β-cyclodextrin and its derivates complexes. The stoichiometries and stability constants of complexes were calculated by the continuous variation and phase solubility methods respectively. NMR is used to confirm the formation of inclusion complexes.

253: Stereoisomeric separation of some flavanones using alpha-cyclosophoro-octadecaoses as a chiral additive in capillary electrophoresis

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α-Cyclosophorooctadecaoses (α-C18), produced by *Rhodobacter sphaeroides*, are mostly homogeneous in size with 18 glucose units per ring as the predominant form. α-C18s are linked by β-(1,4)-linkages and one α-(1,6)-linkage and are also known to be highly substituted by acetyl (0–2 per mol) and/or succinyl groups (1–7 per mol). We isolated and purified α-C18 and successfully used it in capillary electrophoresis (CE) as a chiral additive for the separation of five flavanones and flavanone-7-O-glycosides, including naringenin, hesperetin, eriodictyol, homoeriodictyol, isosakuranetin, and hesperidin. Throughout the CE experiment with unsubstituted α-C18 (uα-C18) obtained after alkaline treatment of the isolated α-C18, we found that successful chiral separation critically depends on the presence of succinate substituents attached to α-C18 in CE, suggesting that succinylation of α-C18 is decisive for effective stereoisomeric separation.

254: Enzymatic Conversion into GalNAc from GlcNAc

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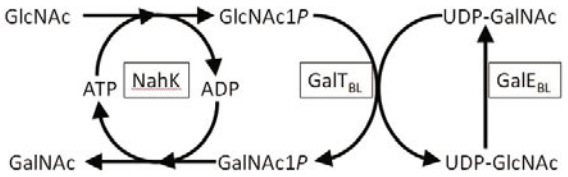
GalNAc is one of the common monosaccharides in the sugar chains of glycoconjugates. Recently, we demonstrated the enzymatic production of GNB (Galβ1,3GalNAc), which is known as the core I disaccharide of O-linked glycoproteins, from sucrose and GalNAc in one-pot. The low availability of GalNAc due to the lack of abundant natural resources makes the procedure not practical. On the other hand, GlcNAc, the C4 epimer of GalNAc is available from chitin, the second abundant polysaccharide next to cellulose in the nature. Thus, we have pursued to develop a practical conversion of GlcNAc into GalNAc. We attempted the enzymatic conversion using the bifidobacterial pathway composed of the following three enzymes that metabolizes GalNAc like a Leloir pathway: (1) N-acetylhexosamine 1-kinase ([EC 2.7.1.162], NahK) that catalyzes phosphorylation of both GlcNAc and GalNAc, yielding their 1-phosphate; (2) UDP-Glc-hexose-1-phosphate uridylyltransferase ([EC2.7.7.12], $GalT_{BL}$) that is also active on N-acetylhexosamine 1-phosphate as a substrate; (3) UDP-Gal/UDP-GalNAc 4-epimerase ([EC5.1.3.2], $GalE_{BL}$). As shown in the figure, GlcNAc is expected to be converted into GalNAc at the presence of catalytic amounts of ATP and UDP-GlcNAc. The key point is the reverse reaction of NahK that GalNAc is produced from GalNAc1P. We confirmed 170mM GalNAc was produced from 600mM GlcNAc at the equilibrium in an actual one-pot reaction, suggesting that the reverse reaction was practically usable. This enzymatic method is a candidate for the practical production of GalNAc from abundant natural sources, and expected to improve the availability of GalNAc used in glycoscience.



255: Synthesis of N-acetyl neuraminic acid (Neu5Ac) by Neu5Ac aldolase protein aggregates.

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N-acetyl-D-neuraminic acid aldolase, a key enzyme in the biotechnological production of N-acetyl-D-neuraminic acid (Neu5Ac) from N-acetyl-D-mannosamine and pyruvate, was characterized as cross-linked by precipitation with 90% ammonium sulfate and crosslinking with 1% glutaraldehyde. Because dispersion in a reciprocating disruptor (FastPrep) was only able to recover 40% of the activity, improved enzymes were then prepared by co-aggregation of the enzyme with 10 mg/mL bovine serum albumin followed by a sodium borohydride treatment and final disruption by FastPrep. This produced a twofold increase in activity up to 86%, which is a 30% more than that reported for this aldolase in cross-linked inclusion bodies (CLIBs). In addition, these FastPrep- protein aggregates presented remarkable biotechnological features for Neu5Ac synthesis, including, good activity and stability at alkaline pHs, a high K_M for ManNAc (lower for pyruvate) and good operational stability. These results open the practicability of using FastPrep protein aggregates for producing Neu5Ac for its use in glycobiology.

This study was partially supported by MEC (BIO2007-62510) and Programa de Ayuda a Grupos de Excelencia

de la Región de Murcia, de la Fundación Séneca (04541/GERM/06, Plan Regional de Ciencia y Tecnología 2007-2010). G.S.C. is a holder of a predoctoral research grant (FPU) from Ministerio de Educación y Ciencia, Spain. M.I.G.G and A.S.C are holders of predoctoral research grants from Grupos de Excelencia de la Región de Murcia, Spain.

256: A new N-Acyl-D-Glucosamine 2-epimerase from *Bacteroides ovatus* with potential biotechnological use.

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Many bacteria have the ability to release sialic acids from complex glycoproteins and oligosaccharides present in the media or on cell surfaces at sites of colonization or infection. To convert one of the released sialic acids, (Neu5Ac) into intermediates that enter the central glycolytic path- ways, Neu5Ac aldolase and than to other bacterial AGEs. Surprisingly, *Bacteroides ovatus* possesses three putative epimerase genes, BACOVA 00274, BACOVA 01795 and BACOVA 01826. Among them, BACOVA 01826 shows a unique long N-terminal region not before described. In this work, we describe the cloning, purification and biochemical characterization of the BACOVA 01826 gene of *B. ovatus* (BoAGE). This enzyme has a broad optimum pH and stability between pH 7.0 and 9.0, and uses ATP as cofactor and stabilizer, as shown from the data obtained from melting curves. BoAGE also interacts with renin, but opposite to eukaryotic renin binding proteins, this interaction produced activation. Phylogenetic analysis of BoAGE sequence describes new subfamilies into bacterial AGEs, not before described. Its potential biotechnological use in the synthesis of Neu5Ac from N-Acetyl-D-glucosamine is also discussed.

This study was partially supported by MEC (BIO2007-62510) and Programa de Ayuda a Grupos de Excelencia de la Región de Murcia, de la Fundación Séneca (04541/GERM/06, Plan Regional de Ciencia y Tecnología 2007-2010). G.S.C. is a holder of a predoctoral research grant (FPU) from Ministerio de Educación y Ciencia, Spain. M.I.G.G and A.S.C are holders of predoctoral research grants from Grupos de Excelencia de la Región de Murcia, Spain.

257: Chemiluminescent visualization and quantification of the complement glycoprotein C4 containing systems in patient whole sera separated with isoelectric focusing in polyacrylamide gel and electroblotted: sialidase- and peroxidase-based new approaches

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Introduction. Glycoprotein C4A and C4B isotypes function as preferential covalent acceptors of protein or carbohydrate targets, respectively. The aim was to develop visually controlled method for patient sera C4 isotype/subisotype deficiency screening using IEF PAG and immunoblotting. Methods: Sera were treated with Clostridium perfringens sialidase (Grade V or VI, Sigma, USA). Then proteins were separated by IEF (pH 3-5 or 4-6) in horizontal 5% PAG block (7 M urea, 5% [w/w] saccharose, 600-700 V, a night, 10°C) followed by electroblotting at pH 8.3. Blots were treated with variants of (anti-humanC4-Ab)-peroxidase. Blot chemiluminescence was monitored in live image regime using BioChemi System (UVP, Calif.). Results: 1. Desialylation of sera with thermostable sialidase was 30-40 minutes at 56°C (complement inactivation) in phosphate buffer (PB) pH 7 [complete desialylation of C4 isotypes in C4A-, C4B-deficient sera used as models] followed by incubation a night at room temperature in the dark [desyalylation of residual sialic acids in C4 isotypes of any patient sera]. Results were in accordance to the developed by us functional C4B/C4A assay in microplate. 2. PAG-obtained blot-1 (pH8 or pH7) was further electroblotted in acetate buffer pH4 or pH3 to get improved blot-1 (pH4) or blot-2 (pH3) [control peroxidase-Ab transfer]. All blots were finally washed with PBS pH7 and PB pH8.5 at 50-55°C without peroxidase inactivation. 3. Sera of patients having autoimmune and infectious diseases were investigated. C4 isotypes were detected within pI 4.0-4.3 and pI 4.6-4.7. Patient sera were grouped as: deficiency of C4B, or C4A, or both C4B and C4A; non-deficiency of C4 isotypes (subgroups C4B > C4A, C4B < C4A). C4A or C4B subisotypes visualized allowed more detailed patient sera C4 system standardization. Conclusions: Methodology developed may be used in case of any sialidase or glycoconjugates; in combination with animal polyclonal and monoclonal Ab, antisera against glycoconjugates. It is also useful for testing of sera desyalylation status. It may be of potential value in identification of sialidase in serum.

258: Porcine submaxillary mucin self interactions and their interaction with soybean agglutinin studied at the single molecular pair level

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Complex oligosaccharides are involved in the control of

many normal and pathological processes. The rich supply of potential low affinity binding sites offered by carbohydrates, arranged in a polyvalent array that may create a flexible and versatile carbohydrate - protein and carbohydrate - carbohydrate recognition system, makes such interactions both interesting and challenging to study. The weak forces involved, combined with the structural heterogeneity of many carbohydrate structures, add to the experimental challenges. However, recent developments within techniques allowing manipulations of single molecules may become important for this field. By these new approaches, the kinetics, mechanics, and variations of structure, function, and interactions can be fully explored by observing how intereaction strengths depend on the force loading rate (i.e. performing force spectroscopy). We have used dynamic force spectroscopy to investigate pairs of soybean agglutinin (SBA) and Tn-porcine-submaxillary mucin (Tn-PSM). The SBA-Tn-PSM unbinding force increased from 103 pN to 402 pN with increasing force loading rate. The lifetime of the complex in the absence of applied force was in the range 1.3 – 1.9 s. Published kinetic parameters describing the rate of dissociation of other sugar lectin interactions are in the range 3.3 x 10^{-3} – 2.5 x 10^{-3} s. The long lifetime of the SBA – TnPSM complex is compatible with a previously proposed "bind and jump" mechanism. This mechanism has also been suggested for lectins binding to multivalent carbohydrates and to multivalent globular glycoprotein. Additionally, we have studied the self interaction properties of mucins with various decoration patterns in order to shed light on the influence of mucin side chain moieties on the interaction properties. Interactions strengths (for most carbohydrate decorations below 50 pN) as well as lifetimes for these interactions were determined. The ability to probe the strength of weak biological intearctions with high specificity and reproducibility open for new insight into the biological functions of i.e. glycoproteins.

259: Medicinal Materials for Diagnostic Imaging and Curing Tumors: Syntheses and Evaluation of Sugar-Ball-Dendritic MRI Contrast Agents and Phospha Sugars

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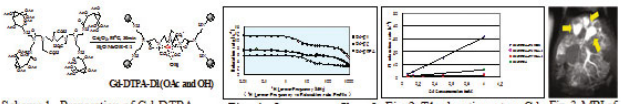
Gd-DTPA derivatives are widely used for MRI contrast agent, however, the agent has poor potentials for imaging blood vessels, cancer cells, etc. To improve MRI contrast agents, sugar dendritic Gd-DTPA complexes, e.g., Gd-DTPA-D1-Glc(OAc), were prepared and/or hydrolyzed to give Gd-DTPA-D1-Glc(OH) (DEN-OH) (Scheme 1) [1]. The in vitro evaluation of the sugar dendritic Gd-DTPA complexes showed preferable T1 relaxation rates vs Larmor frequency (Fig. 1) and Gd-concentration (Fig. 2), which revealed that DEN-OH must be a superior MRI contrast agent to Magnevist. The in vivo evaluation of DEN-OH showed an excellent MRI imaging of liver cancer cells compared with Gd-DTPA (Fig. 3).

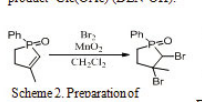


Scheme 1. Preparation of Gd-DTPA-D1-Glc(OAc) and/or hydrolyzed product -Glc(OAc) (DEN-OH).

Fig. 1. Larmor profile of sugar dendritic Gd-DTPA complexes.

Fig. 2. T1 relaxation rate vs Gd concentration of Gd-DTPA complexes and DEN-OH.

Fig. 3. MRI of liver cancer by DEN-OH.



Scheme 2. Preparation of

Fig. 4 Phospha sugars 1 (Top) and 2 (Bottom)

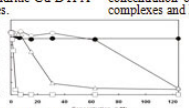


Fig. 5 MTT assay of phospha sugars (BMPP (○), DBMPP (△), and TBMPP (□)) against leukemia cells (U937).

Oxidation or addition reaction of 2-phospholenes with OsO_4 or Br_2 in aqueous organic medium afforded cis-diol 1 or bromohydrine 2 (Fig. 4) of tetrofuranose type phospha sugars. The catalytic addition reaction of 3-methyl-1-phenyl-2-phospholene with Br_2 in organic medium afforded dibromodi-deoxyphospha sugar analogue (DBMPP) (Scheme 2). The MTT in vitro evaluation of these phospha sugar analogues showed higher potential for anti-tumour agent against leukemia cells than Gleevec (Fig. 5) by inducing apoptosis [2]. The phospha sugars are effective against solid tumour cells. Acknowledgement: Financial supports by the research funds of MHLW, MEXT, JSPS, and JST of the Japanese Government are greatly acknowledged.

References: [1] M. Takahashi, Y. Hara, K. Aoshima, M. Yamashita, et.al., Tetrahedron Lett., 41, (2000) 8485-8488. [2] S. Nakamura, M. Yamashita, D. Yokota, M. Fujie, et. al., Invest. New Drugs, 28, (2010), 381-391.

Poster Session I (Monday)

Infection & Immunity

260: Modulation of the expression of *Helicobacter pylori* glycan receptors in gastric mucosa

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The gastric pathogen *Helicobacter pylori* exploits the glycans expressed on the surface of the host cells to colonize the gastric mucosa [1]. Infection by *H. pylori* is associated with peptic ulcer and gastric cancer development. The bacterial adhesion to the gastric cells constitutes a key step to a successful infection, thus understanding the molecular mechanisms underlying the expression of the bacteria ligands is of utmost importance. The initial steps of adhesion are mediated by the blood group antigen binding adhesin (BabA), which binds to the fucosylated H-type 1 and LewisB antigens. It has been suggested that H. pylori is able to modulate the host glycosylation profile, taking benefit of newly expressed sialylated antigens, which are recognized by a Sialic acid binding adhesin (SabA). We have previously demonstrated, using gastric cell lines, that H. pylori is able to induce the expression of β3GnT5, that participates in the synthesis of sialyl-LewisX, leading to increased SabA-mediated bacterial adhesion [2]. This study aims to characterize the glycosylation profile of gastric mucosa of *H. pylori* infected individuals considering the expression of specific glycosyltransferases, the glycan phenotype and the adhesins expression profile of the infecting *H. pylori* strains. We have observed increased expression of the sialylated receptors sialyl-LewisA and sialyl-LewisX in the gastric mucosa of *H. pylori* infected individuals. Approximately 50% of the *H. pylori* strains isolated from the same individuals expressed the SabA adhesin. We are presently evaluating the expression of candidate glycosyltransferases genes that may account for this altered gastric glycophenotype promoted by *H. pylori* infection.

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261: Fucose-containing bacterial capsule in bacterial virulence

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Klebsiella pneumoniae (KP) contains thick capsular polysaccharides. KP belongs to Enterobacteriaceae and is considered as one of the normal flora in human gut. Emerging clinical disease liver abscess (LA) caused by KP indicates increased bacterial virulence. Examination of bacterial capsular composition revealed that LA-KP and UTI-KP (causes urinary tract infection but not liver abscess) have similar composition except LA-KP contains large amount of fucose while UTI-KP contains high amount of mannose, but no fucose. In bacteria, mannose is converted to fucose by two genes, gmd and wcaG. All UTI-KPs screened did not possess these two genes whereas most of the LA-KPs contained both genes. Knock out gmd gene in LA-KP abolished virulence in infected mice while the wild type caused high death rate. Mutant and UTI-KP interacted with macrophages readily while wild type KP did not, indicating mannose could be exposed on bacterial surface in mutant and UTI-KP as macrophages contain mannose receptors. Fucose-containing capsule on LA-KP enables the bacteria to evade host immune clearance. The bacterial infections increased blood IL-1b level, but not TNF-a in mice. HepG2 cells infected with LA-KP activated TLR1/2, TLR4 and the downstream MEK1/2-ERK-p90RSK and MKK3/6-p38 kinase-ATF2 activations, but not JNK pathway. Infections also caused DNA laddering at and after 6 hour post infection, although early apoptosis and late necrosis were observed by flow cytometry. Curiously, caspase 3 was not involved during this infection. Mitochondria released endonuclease G and DNA fragmentation factor (DFF)40, released from inhibitor DFF45 contribute to DNA laddering. These DNA damages trigger poly(ATP-ribose)polymerase (PARP) activation for repair and eventual ATP depletion. Both necrosis markers, PARP and Calpain were activated, however the smearing of DNA degradation of necrosis was not observed, instead DNA laddering persisted till the end. Whether the fucose content contributes to this phenomenon is not known.

262: New pH dependent lectin from bioluminescent bacterium *Photorhabdus luminescens*

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The carbohydrate structures located on cell surface serve as recognition sites between a pathogenic bacterium and a host cell. Lectins, carbohydrate-binding proteins, play an important role in cell recognition and adhesion in the first step of their invasion and infectivity. *Photorhabdus luminescens* (formally *Xenorhabdus luminescens*) is a gram-negative symbiotic bacterium that lives in the gut of entomopathogenic nematodes. It is the bioluminescent pathogen of insect. The protein PLU from *P. luminescens* has a strong sequence homology with the recently characterized lectin BC2L-C-nt from the human opportunistic pathogen *Burkholderia cenocepacia*. BC2L-C-nt is an attractive protein, since it has unique β sandwich trimeric structure and is the first lectin with TNF-fold[1]. The contribution is focused on characterization of lectin PLU from *P. luminescens*. The gene coding the protein was cloned and expression and purification conditions

were optimised. Function studies were performed using surface plasmon resonance and isothermal titration calorimetry, which allowed identifying binding properties and thermodynamic parameters of interaction with saccharides. Oligomeric state of protein was determined using dynamic light scattering, crosslinking with bifunctional reagents and analytical ultracentrifugation. The results indicated the unusual activity of the protein. The protein is active in a very narrow interval of pH and small pH changes lead to a protein aggregation. This work is supported by Ministry of Education (MSM0021622413, ME08008) and Grant Agency of Czech Republic (GA/303/09/1168, GD301/09/H004). [1]. Šulák O., Cioci G., Delia M., Lahmann M., Varrot A., Imberty A., Wimmerová M., Structure, 2010, 18, 59-72.

263: *Burkholderia cenocepacia* lectins – similar, however different potential virulence factors

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Burkholderia cenocepacia is a Gram-negative bacterium causing a number of diseases in plants but this micro-organism also infects immunocompromised humans, especially cystic fibrosis (CF) patients. Chronic pulmonary colonization by opportunistic bacteria is the leading cause of death among humans suffering from CF. Crucial role in the infection could be played by bacterial lectins. Microbial lectins can recognize carbohydrates on host cells (glycoproteins or glycolipides) and mediate adhesion to host cells or mucosal surfaces. Adhesion is considered to be an important step in infection development as it protects pathogen from natural cleansing mechanisms of the host. *B. cenocepacia* genome contains three to four (in dependence on a strain) homologues of lectin PA-IIL from *Pseudomonas aeruginosa,* the most widespread pathogen associated with cystic fibrosis. Our work is focused on characterization of lectins from *B. cenocepacia*, especially on determination of their structure and binding properties. As blocking of lectins with potent inhibitors could decrease adhesion of pathogens to host cells, these proteins are potential candidates for an anti-adhesion therapy. Therefore, thermodynamics of binding, affinity and specificity of the lectins was determined by surface plasmon resonance and isothermal titration calorimetry. In contrast to PA-IIL, majority of *B. cenocepacia* lectins preferentially recognizes D-mannose and mannosides but these proteins also bind glycero-D-manno-heptose disaccharides and heptose derivatives. Thus, lipopolysaccharides from *B. cenocepacia* could be suitable targets for lectins and anchor them on the bacterial surface. Although structures of these proteins are highly similar (as was determined by crystallographic methods) they significantly differ in binding affinity and thermodynamical properties. Despite of being homologues, they probably execute different functions in pathogenesis or are expressed in diverse stages of infection.

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264: Uptake of oligomannose-coated liposomes by mouse peritoneal macrophages promote a differentiation of the cells into dendritic cells

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Dendritic cells (DCs) are potent professional antigen-presenting cells that capture antigens and migrate to T cell regions of draining lymph nodes, where they initiate primary immune responses. It has been shown that mouse resident peritoneal macrophages as well as monocytes are potent progenitor cells for DCs. On the other hand, we have demonstrated that oligomannose-coated liposomes (OMLs) are preferentially and rapidly taken up by mouse resident peritoneal macrophages, and that OMLs can induce antigen-specific Th1 immune responses and cytotoxic T lymphocytes, if antigen-encased OMLs are administrated into peritoneal cavity. Therefore, it may be possible that the peritoneal macrophages differentiate into mature DCs in response to OML uptake to initiate the specific immune responses. In this study, we demonstrate that mouse resident peritoneal macrophages can differentiate along a DC lineage in response to preferential uptake of OMLs. When OMLs were administered into the peritoneal cavity, about 30% of the resting peritoneal cells preferentially and rapidly took up OMLs. The cells with ingested OMLs expressed a high level of CD11b, CD14 and F4/80, but lacked expression of CD11c, indicating that these OML-ingesting cells with a CD11b(high) CD11(-) phenotype belong to a monocyte/macrophage lineage and are usually referred to as the resident peritoneal macrophages. The CD11c(+) cells then arose among the peritoneal cells with ingested OMLs during in vitro cultivation for 24 h. When the CD11b(high)CD11(-) cells were enriched as adherent cells from OML-treated mice, the CD11b(high) CD11c(+) cells also developed among the enriched fraction within 1 day, and the resulting CD11c(+) cells expressed significant levels of CD40, CD86, CCR7 and MHC class II. In addition, CD11b(high)CD11c(+) cells with ingested OMLs were found in spleen after the enriched peritoneal CD11b(high)CD11(-) cells with ingested OMLs were transplanted in the peritoneal cavity of mice. These results show that a part of the resident peritoneal macrophages can differentiate into mature and functional DCs following uptake of OMLs, leading induction of the characteristic immune responses, if the mice receive OML in the peritoneal cavity.

265: Differential responses of NKT cells against different types of α-GalCer-containing liposomes

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A subpopulation of T cells called NKT cells recognize glycolipids antigens presented by the MHC class I-like molecule CD1d. NKT cells regulate a number of critical biological conditions in vivo through the secretion of Th1 and Th2 cytokines. Alpha-GalCer has been used as an exogenous ligand for CD1d to stimulate NKT cells, and is currently being tested for cancer immunotherapy. Since α-GalCer is very strong ligand that stuns the NKT cells to lead to anergy if α-GalCer is directly administrated in vivo, a successful NKT cell immunotherapy approach is to induce mature dendritic cells (DCs) pulsed with α-GalCer ex vivo followed by injection of α-GalCer-loaded DCs into patient to expand NKT cells. However, isolation of DCs followed by pulsing with antigen is impractical. Specific delivery of α-GalCer to DCs in vivo may be one of the promising ways to overcome these problems. We have shown that the liposomes coated with a mannotriose-containing neoglycolipid (Man3-DPPE) can deliver the encapsulated proteins to DCs effectively and induce the antigen-specific cellular immune responses. Therefore, we presume that Man3-DPPE coated liposomes can be used as a vehicle for specific delivery of lipid antigens to DCs. In the present study, we examined in vitro response of mouse splenocytes toward α-GalCer-containing liposomes coated with Man3-DPPE (GCM3L) or without Man3-DPPE (GCL). The splenocytes in vitro stimulated with GCM3L produced considerable amounts of IFN-gamma. The level of IFN-gamma produced by GCM3L-stimulated splenocytes was similar to that by splenocytes stimulated with soluble α-GalCer. In contrast, the IFN-production level of GCL-stimulated splenocytes was significantly lower than that of GCM3L-stimulated splenocytes. On the other hand, the levels of IL-10 and IL-4 produced by GCL-stimulated splenocytes were similar to those by GCM3L-stimulated splenocytes. Depletion of either CD3(+)-, CD5(+)-, DX5(+)- or CD11c(+)- cells from splenocytes abolished the production of these cytokines, suggesting that both NKT cells and DCs were involved in these responses. We also found that GCM3L was incorporated into CD11c(+)cells much more effectively than GCL. These results suggest that the different preference of liposomal uptake by splenic DCs induce different responses of NKT cells, and GCM3L can lead to production of the Th1 cytokine much more predominantly over the Th2 cytokine than GCL or soluble alpha-GalCer. Our results also suggest that GCM3L can be potentially used for preferential delivery of the lipid antigens to DCs in vivo to activate NKT cells.

266: Glycosylation patterns of Immunoglobulin G following group B *Neisseria meningitidis* vaccination of human volunteers using LC-MS-ESI Orbitrap

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Meningococcal disease is caused by a Gram-negative diplococcus bacteria, *Neisseria meningitidis*. Thirteen serogroups of *N. meningitidis* have been identified, and vaccines have been developed against some of these. An outer membrane vesicle vaccine, MenBvac® against serogroup B meningococcal disease has been developed at the National Institute of Public Health in Oslo. A similar vaccine against the New Zealand strain, NZ, has been developed by Chiron-Novartis and the University of Auckland, MenZB™. The MenBvac® vaccine has been extensively tested and plasma samples from different time points following vaccination have been analysed. Our areas of interest have been the IgG glycosylation patterns, and immunoglobulin subclass distribution. Variation in carbohydrate structure attached to asparagine 297 can cause differences in interaction with Fc receptors, and hence provide different levels of protection against meningococcal disease. The lack of core fucose on N-IgG glycans have previously been reported to enhance antibody dependent cell mediated cytotoxicity (ADCC). The presence of sialic acid on N-IgG glycans have been shown to provide antibodies with anti-inflammatory properties, while the lack of sialic acid causes pro-inflammatory properties. Mass spectrometry applying a LC-MS-ESI Orbitrap system was used to determine the glycosylation patterns. The plasma samples used were taken at three different time points, the first two following 3 doses of the vaccine, and the third time point was after the fourth dose. Some volunteers were vaccinated with MenZB™, and some were vaccinated with one vaccine for the first 3 doses, and the other vaccine for the fourth dose. Determining the concentrations of the different IgG subclasses can together with the glycosylation patterns provide a better understanding of the specific structure of the antibodies produced at different times following immunization. Put together with biological assays like bactericidal and opsonophagocytic activities to determine in vitro surrogate levels of protection, this information can be used to create more efficient vaccines or vaccine schedules in the future.

267: Modulation of the biological function of a synthetic carbohydrate antigen related to *Streptococcus pneumoniae* infections

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Capsular polysaccharides (CPS) are important virulence factor of invading bacteria. Antibodies to capsule are, in fact, highly protective against the invasive disease, and CPS formed the basis for the development of pneumococcal vaccines. However, there are several problems associated to the preparation of a highly effective vaccine. One of the most important issue is to establish strategies to overcame the poor immunogenicity and T-independent nature of saccharidic antigens. The T-lymphocyte independent nature of a polysaccharide may be overcome by conjugating the native or depolymerized polysaccharide to a carrier protein. In a similar way, the conjugation to an immunoadjuvant could lead to a construct able to stimulate a sustainable antibody response. Recently, lipoproteins, i.e. Pam3Cys, have been shown as potent activators of B-lymphocytes and macrophages, and can be considered as interesting immunoadjuvants to be used as tools for the development of conjugated vaccines. Herein, the synthesis of a glycoconjugate where the repeating unit of *Streptococcus pneumoniae* (SP) type 19F capsular polysaccharide is linked to a Pam3CysSer residue through an amino propyl linker will be presented. The final aim is to evaluate the influence of the lipopeptide on the immune response towards SP, and if antigen specific antibodies can be elicited with low molecular weight conjugates of Pam3CysSer with a saccharide-based hapten.

268: Design, Preparation and Preliminary Evaluation of Glycoconjugate Vaccines Active Against Anthrax

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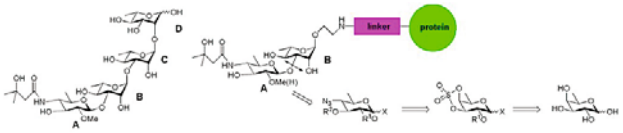
Bacillus anthracis, the etiological agent of anthrax, is currently one of the main bioterrorist threats. This has stimulated the development of effective vaccine against anthrax. Along this line, the recent disclosure of a tetrasaccharide (Scheme), displayed on the outermost surfaces of *B. anthracis* spores, has suggested the possible development of a glycoconjugate vaccine, known to be safe and efficient in humans. This tetrasaccharide, and mainly the A residue which is specific of *B. anthracis* and some related *Bacillus* species, has been shown to be one of the targets of the immune response in mice and rabbits. On the basis of these observations, we have designed the preparation of glycoconjugate vaccines derived from disaccharide AB. The preparation of the different anthrose-containing carbohydrate moieties of the glycoconjugates relies on a new synthetic route. This strategy takes advantages of cyclic sulfite/sulfate intermediates which serve successively as protecting and as leaving groups. The conjugation of these disaccharides to a carrier protein as well as to T-helper epitopes and the preliminary evaluation of the vaccine potency of these glycoconjugates will be presented as well.



Scheme. Structure of the native tetrasaccharide of the exosporium glycoprotein of *B. anthracis* and retrosynthetic strategy of anthrose-containing conjugates

269: Characterisation of Pathogen Associated Molecular Patterns isolated from the causative agents of bovine mastitis

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Bovine mastitis is an inflammatory response of the mammary gland to bacterial infection that affects dairy cows. Major causative agents are *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *St. dysgalactiae*. *E. coli* elicits acute inflammation whereas the other species usually cause subclinical inflammation. Lipopolysaccharide (LPS, *E. coli*) and lipoteichoic acid (LTA, *Staphylococcus* and *Streptococcus*) represent Pathogen Associated Molecular Patterns which evoke host immune responses. Most LPS comprise the O-antigen, the core and lipid A. LTA contains an often substituted 1,3-polyalditolphosphate linear chain and a glyceroglycolipid anchor. LPS were isolated from *E. coli* 1303 and P4 acute mastitis strains and the structures of the O-antigens were characterized by mass spectrometry as well as NMR spectroscopy. LTA was isolated from three mastitis *S. aureus* clonal lineages after n-butanol extraction, and the structures were characterized by compositional analyses and NMR spectroscopy. Additionally, structural analyzes were performed on the LTA lipid anchor from *St. dysgalactiae* which was isolated after HF treatment and $CHCl_3$ extraction. The immunostimulatory capacity of those isolates was tested in primary bovine Mammary Epithelial Cells (pbMEC) which were treated for different periods (1, 3, 24 h) with different concentrations of LPS (10, 100 ng /ml) or LTA (1, 10μg/ml). qRT-PCR was used to measure the mRNA concentrations of various immune relevant genes. Several of these genes were differentially induced by the two different ligands. LPS induced very strongly the mRNA concentration of the chemokine CCL5 (360 fold), while 10 μg/ml of LTA induced this only ~10 fold, thus to less than 3% of the level of LPS caused induction. In contrast, LTA induced the concentration of TNF-a ~ 100 fold and thus just to the same extent as it was induced by LPS. These data reveal that the MEC differentially induces

immune relevant genes in a PAMP specific fashion. (Supported by the DFG through the FOR585; project 8 & Se 326/14-3).

270: Reishi Polysaccharides-induced Antibodies Recognize Tumor- Associated Carbohydrate Epitopes

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Detailed evaluation of Reishi (*Ganoderma lucidum* or Ling-Zhi, a medicinal fungus) is challenging. While Reishi has been used for thousands years by people all over the world to treat disease and promote health, the exact mechanism of action is still not well understood. The saccharide portion of glycoproteins and glycolipids of Reishi contains a wide variety of immunogenic epitopes that may induce predominantly antibody responses. Therefore, the carbohydrate moieties of Reishi are thought to be important antigenic determinants. By applying carbohydrate microarray analysis, we show here for the first time that antibodies to a cancer associated carbohydrate epitope, Globo H, are induced in mice after immunization with a fucose-containing fraction of Reishi (FFOR), thereby exerting anticancer activity. This is a previously unrecognized but important mechanism. A more exciting finding is that immunostaining of a FFOR with the known anti-Globo H monoclonal antibodies, VK9 and MBr-1, suggests that a tumor-associated carbohydrate epitope "Globo H-like" structure existing in FFOR. We anticipate that our results would lead to the development of Reishi polysaccharides as cancer vaccines capable of inducing epitope-specific anti-cancer antibodies and contribute our understanding of FFOR-mediated anti-cancer activity. Furthermore, we demonstrate the utility of carbohydrate microarrays for evaluating immune responses to large, complex immunogens such as herbal medicine and the ability to detect previously unrecognized epitopes.

271: Analysis of Chicken Intestinal Mucin O-glycans by LC-MS: Potential Inhibitory Elements for *Campylobacter jejuni* Infection.

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Colonization of *Campylobacter jejuni* in the human intestinal track can cause gastroenteritis and in severe cases to Guillain-Barré syndrome (GBS). The bacteria are commensal in poultry, residing in the mucosal layer but not adhering or invading the epithelial cells. Mucin from chicken large intestine attenuates binding of *C. jejuni* better than the small intestine with the mechanism of inhibition being attributed to mucin glycosylation. Previous reports have demonstrated that particular glycan epitopes in human milk, namely α1,2-fucosylated glycans inhibit colonization. Additionally, sulphation functions to decrease susceptibility to *C. jejuni* infection. However the exact mechanisms responsible for binding and colonization are unknown but glycans unique to chicken large intestine may provide useful inhibitory structures to be used in reducing the incidence of campylobacteriosis worldwide. Here we present a detailed structural analysis of O-glycans released from purified chicken mucin large and small intestines by negative ion LC-MS^n.

272: Characterization of a novel glucosyltransferase WbdN from *Escherichia coli* serotype O157

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Lipopolysaccharides (LPS) are amphiphilic macromolecules on the surface of Gram-negative bacteria, and are important for growth and viability of bacteria. The outer portion of LPS is the O-antigen which consists of repeating units of oligosaccharides. The enterohaemorrhagic O157 strain of *Escherichia coli* has an O-antigen repeat unit with the sequence [→2-D-Rha4NAcα1→3-L-Fucα1→4-D-Glcβ1→3-D-GalNAc1→]. We proposed that the wbdN gene found in the O-antigen gene cluster of *E. coli* O157 encodes a β1,3-glucosyltransferase that adds Glc in β1-3 linkage to GalNAc as the second step in O-antigen repeat unit synthesis. The wbdN gene was expressed in *E. coli* BL21 bacteria, and WbdN activity was characterized using the donor substrate UDP-[^{14}C]Glc and the synthetic acceptor substrate GalNAcα-PO_3-PO_3-$(CH_2)_{11}$-O-Phenyl. The resultant disaccharide product was isolated by HPLC. EI-MS (negative ion mode) showed that one Glc residue was transferred by WbdN. WbdN has a DxD motif and requires divalent metal ions for full activity, with Mn^{2+} ion being the best activator. Mg^{2+} and Co^{2+} also activate the enzyme. WbdN activity has a broad pH optimum, is stable at both 4°C and -20°C, and is highly specific for UDP-Glc as the donor substrate. GalNAcα derivatives lacking the pyrophosphate group were inactive as substrates. Our results illustrate that WbdN is a UDP-Glc: GalNAcα-pyrophosphate-lipid glucosyltransferase that is different from previously studied Glc-transferases and has very little sequence similarity to other glycosyltransferases. This work was supported by NSERC.

273: Cloning, Expression and Characterisation of Glycosyltransferases from the Mosquito *Anopheles gambiae*

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The mosquito *Anopheles gambiae* is the primary vector of human malaria. Two major tissues and two fundamental steps within the mosquito are required for the parasite development and transmission. First, the Plasmodium ookinete stage is recognized and attached to luminal membrane ligands of the mosquito midgut epithelium prior to invasion. The second step is the transmission of the sporozoites into a new vertebrate host. Both steps, binding of parasite in insect vector and in vertebrate host cells, have been connected to the presence of oligosaccharide structures (glycans).

As the *A. gambiae* genome was published recently, three fucosyltransferases and one peptide-O-xylosyltransferase were found. By homology searching *A. gambiae* has a Lewis type, a core α1,3 and a core α1,6 fucosyltransferase. All three fucosyltransferases as well as the peptide:O-xylosyltransferase were cloned with a FLAG-tag into pPICZαC vectors and transformed in *Pichia pastoris* GS115 cells using zeocin selection. The recombinant core alpha 1,6 fucosyltransferase (AgFucT6) was expressed and purified by Affi-Gel Blue affinity chromatography. The characterisation of AgFucT6 shows that in comparison to many other glycosyltransferases, the mosquito core α1,6 fucosyltransferase has no absolute requirement for any special divalent cation.

The mosquito core α1,6 fucosyltransferase was then used in an apo-transferrin remodelling experiment to create positive controls for Lectin Blot analysis. We are able to modify at least one of the two N-glycosylation sites in human apo-transferrin which can be confirmed with AAL (*Aleuria aurantia* lectin) binding to the remodelled transferrin sample. The peptide-O-xylosyltransferase (AgOXT1) was also expressed and characterised; in MALDI-TOF MS and HPLC-based assays various optima were determined as well as its K_m value for the UDP-Xyl donor. In other in vivo experiments, RNAi of AgOXT1 in the midgut disrupted ookinete invasion, whereas RNAi in the salivary glands of the mosquito indicates a role for heparan sulphate in sporozoite invasion.

274: Human synovial lubricin has L-selectin ligand activity and associates with neutrophils

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Synovial fluid is responsible for lubricating the joint, which is the primary tissue for symptoms of osteoarthritis (OA) and rheumatoid arthritis (RA). Lubricin is an abundant mucin-like glycoprotein in synovial fluid. Except lubricating and protection of cartilage, lubricin also posses other biological functions such as chondroprotection and control of synovial cell growth. In this study, O-linked oligosaccharides from lubricin isolated from OA and RA synovial fluid were investigated by LC-MS. O-glycans on lubricin were found to be mainly sialylated core 1, but small amount of sialylated core 2 structures, containing both sulphate and fucose, was found . The presence of sulphated and fucosylated O-glycans on lubricin imply that its glycosylation may have specific function in addition to the lubricating property provided by simple core 1 structures. Further study showed L-selectin present on human neutrophils was able to bind to synovial lubricin. This indicates that lubricin may be involved in inflammatory cells recruitment influencing the L-selectin facilitated tethering.

275: Human Intelectin interactions with protozoan parasites.

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Intelectin I (ITL-1) is a soluble innate immune lectin that has a Ca^{2+} dependent affinity for D-pentose and D-galactofuranosyl residues present in bacterial and fungal cell walls and some intestinal parasites like *Trichinella* and *Nippostrongylus*, but not in mammalian cells, suggestive of a role in pathogen recognition. Recent evidence also points a suggestive role in allergic inflammation. However, information on the role of hITL-1 on infections caused by intracellular parasites like *Trypanosoma cruzi* or *Leishmania* is absent. Here we present evidence that human ITL-1 specifically interacts with infective metacyclic trypomastigotes from T. cruzi regardless of the genetic lineage of the parasite. In contrast, we found that hITL-1 interacts with much greater affinity with cutaneotropic *Leishmania* (*L. braziliensis, L. tropica* and *L. major*) promastigotes than with those exhibiting viscerotropic properties (*L. infantum* and *L. donovani*). We have followed the course of ITL-1(a) gene expression upon experimental murine *Trypanosoma cruzi* infection in susceptible (Balb/c)and resistant (C57/BL6) model up to 60 dpi. The results show a significant upregulation of ITL-1 gene expression in the heart of susceptible animals (that closely parallels parasitaemia), that last for the first 28 days pi; with no change observed on the resistant model.

We do not have evidence at this moment that allows us to rule out infiltration of macrophages or APCs as the cause of the increased expression. No significant changes were observed in spleen or thymus tissues. Curiously, in genetically deficient animal models for galectin-3 (in C57/BL6 resistant background), we have found a pattern of ITL-1 expression similar to the observed on susceptible model. Implications of the potential relationship between different families of innate immunity molecules will be discussed on the context of parasite infections, as well as the implications of different exposure of ITL ligands on *Leishmania* species causing different pathologies.

276: Modulation of Leishmania GIPLs Synthesis by Myriocin. Parasite Growth, Morphology, and (Glyco) lipid Expression

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Studies carried out in our lab clearly demonstrated that glycosylinositolphospholipids (GIPLs) and (glyco) sphingolipids are present in detergent resistant membrane microdomains (DMRs) of *Leishmania (Leishmania) amazonensis* and *Leishmania (Viannia) braziliensis*, and that DMRs organization has a central role in the parasite infectivity. In the present work it was evaluated the effect of myriocin, an inhibitor of serine palmitoylserine transferase in parasite growth, infectivity and (glyco)(sphingo)lipid expression in promastigote forms of *L. amazonensis* and *L. braziliensis*, which are responsible, respectively for cutaneous and mucocutaneous leishmaniasis in New World. Analysis of lipid extracts by high performance thin layer chromatography showed that myriocin promotes in both *Leishmania* species a drastic reduction of inositol phosphorylceramide expression, the major sphingolipid expressed in promastigote forms together with an increase of GIPLs expression (2-4 times). Variation in phospholipid expression was observed when promastigotes were incubated with low concentration of myriocin. In *L. amazonensis* myriocin 2.5 μM led to significant increase of phosphatidylserine and of lyso-phosphatidylinositol (about 17% and 5.5%, respectively). In *L. braziliensis* myriocin 1 μM increased about 4.5% phosphatidylserine expression. Moreover, myriocin was able to inhibit promastigotes growth in a dose dependent way for both species of *Leishmania*. Myriocin 24 μM inhibited completely *L. amazonensis* growth, without affecting the parasite viability. On *L. braziliensis* myriocin 5 μM lead to the appearance of promastigostes with 3 nuclei and 3 kineplatoplasts (3N3K) and higher concentration of myriocin led to formation of higher polinucleated still unidentified forms, suggesting that reduction of sphingolipids followed by significant increase of GIPLs synthesis may inhibit *L. braziliensis* cytokinesis. Myriocin (12 μM) was also able to promote a remarkable decrease (about 77%) in the infection rate of murine macrophages by L. amazonensis promastigotes. Myriocin effect on parasite glycolipid synthesis, cell biology, vesicle trafficking, as well as organization of membrane microdomains are under investigation. Supported by FAPESP, CAPES, CNPq

277: Human GII.4 norovirus VLP induces membrane invaginations on giant unilamellar vesicles containing secretor gene dependent α1,2-fucosylated glycosphingolipids

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Norovirus is a non-enveloped virus causing acute gastro-enteritis. For human norovirus, no cell culture system is available and consequently knowledge on cellular entry of the virus is limited. The virus binds to ABH histo-blood group glycans on glycoproteins and glycoshingolipids (GSLs). Non-secretor individuals, characterized by a lack of ABH histo-blood group glycans in the gastrointestinal tract, are resistant to most norovirus infections, suggesting that these glycans may be part of the viral receptor. Recent studies have shown that polyomavirus enters the cell via membrane invaginations induced by the multivalent binding of the virus to receptor GSLs. In this study, we have investigated whether norovirus has the ability to induce membrane invaginations on giant unilamellar vesicles (GUVs) containing purified GSLs. The GUVs were composed of DOPC (64 mol %), cholesterol (30 mol %), BodipyFL-C5-HPC (1 mol %, fluorescent, green) and GSL (5 mol %). Virus like particles (VLPs) from the Dijon strain, belonging to the clinically dominant genogroup II.4, were fluorescently labelled with Cy3 (red). In analogy with thin-layer chromatography experiments, the VLPs recognized GUVs containing ABH active GSLs (H type 1, Lewis b, B type 1 or A Lewis b), but not GUVs containing GSLs typically found in non-secretor individuals (lactotetraosylceramide or Lewis a). Upon binding to the vesicles, the VLPs formed highly mobile clusters on the surface of the GUVs. Progressively VLP containing tubular invaginations were seen on the GUVs containing binding GSLs. The VLPs did not bind, nor induce any invaginations on the lactotetraosylceramide or Lewis a containing GUVs. The incorporation of the Lewis a GSL into the GUVs was confirmed using a monoclonal antibody. The antibody recognized the antigen, but did not induce invaginations. In conclusion, this study suggests that human norovirus has the ability

to induce membrane curvature by binding to and cluster GSLs, which may reflect the first step in cellular entry of the virus.

278: Impact of Influenza A virus hemagglutinin N-glycosylation on immunogenicity

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Glycosylation of influenza virus proteins is host dependent and can influence protein properties such as structural stability, protease resistence, or biological activity. It has already been reported that hemagglutinin (HA) from the influenza A H1N1/PR/8/34/RKI strain propagated in various cell lines exhibits distinct N-glycosylation patterns. However, it is still discussed whether and in which way differential glycosylation affects immunogenicity of the virus. In this study, we investigated whether the difference in the N-glycosylation pattern of influenza A virus produced in MDCK cells (M-variant) or Vero cells (V-variant) had an impact on virus immunogenicity. For this purpose, a stimulation assay using TCR-HA transgenic spleen cells was performed. All T cells from TCR-HA transgenic mice have an αβ T cell receptor specific for the peptide 110-120 from influenza HA presented by I-E^d MHC class II molecules. In this assay, a significant difference in the up-regulation of the T cell activation marker CD69 and in cytokine production was observed. Spleen cells incubated with the V-variant up-regulated CD69 faster and to a significantly higher extent than splenocytes incubated with the M-variant. Additionally, the V-variant led to a significantly increased secretion of IL-2. In contrary, spleen cells incubated with the M-variant secreted higher levels of effector cytokines such as IL-4 and IFN-γ. The difference in the immunostimulatory effect can be due to a differential uptake and/or processing of virus derived proteins or recognition by different lectin receptors leading to the activation of distinct signaling pathways. In conclusion, we show that virus protein glycosylation has a significant effect on immunogenicity. This finding might have important implications for influenza vaccine manufacturing. In vivo immunization studies with TCR-HA transgenic mice are currently ongoing.

279: Towards a new synthetic carbohydrate-vaccine candidate based on gold glyconanoparticles

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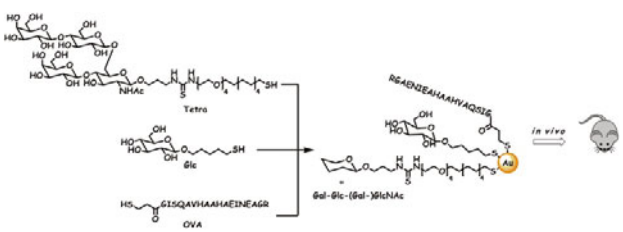
Since carbohydrates are usually poorly immunogenic, strategies have been developed to improve their immune response. [1] In search for new conjugate vaccines not based on protein carriers, we here present gold glyconanoparticles (GNPs) as a versatile platform to construct a potential carbohydrate-based vaccine against *Streptococcus pneumoniae* type 14 (the major cause of acute respiratory bacterial infections). Gold surface can be simultaneously tailored with different ligands in a controlled way via thiol chemistry affording multivalent and multifunctional nanoparticles.[2],[3] The branched tetrasaccharide Gal(β1-4)Glc(β1-6)[Gal(β1-4)] βGlcNAc, corresponding to the repeating unit of the pneumococcal type 14 capsular polysaccharide (Pn14PS), was selected as the smallest structure able to evoke antibodies against bacterial capsular polysaccharide.[4] Antigenic tetrasaccharide Gal(β1-4)Glc(β1-6) [Gal(β1-4)]βGlcNAc, ovalbumin peptide-fragment $OVA_{323-339}$ which is well known as immunodominant T-cell epitope, and glucose were suitably functionalised with different thiol-ended linkers and employed as ligands for the construction of hybrid GNPs. These GNPs, having mean gold core diameters of ~2 nm, were used to immunise intracutaneously BALB/c mice. Depending on the density of the different ligands, GNPs coated with the tetrasaccharide conjugate are capable of inducing specific IgG antibodies against the native polysaccharide of S. pneumoniae type 14. Although further optimization is necessary, this study presents the first example of a fully synthetic carbohydrate vaccine candidate based on nanoparticles. These results should encourage the use of gold GNPs as new systems in the development of a synthetic carbohydrate-based pneumococcal vaccine. [1] Nat. Rev. Drug Discov. 9, (2010) 30 [2] Chem. Eur. J., 9, (2003) 1909 [3] Carbohydr. Res., 342, (2007) 448 [4] Infect. Immun., 70, (2002) 5107



280: Development Strategies of Bacterial Polysaccharide Glycoconjugate Vaccines

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Purified bacterial capsular polysaccharides have been used as vaccines for decades. The resulting immune responses elicited from these polysaccharide vaccines, however, have often been less than satisfactory, especially in young children. Conjugation of capsular polysaccharides to immunogenic protein carriers, on the other hand, has successfully been used to overcome this inherent lack of polysaccharide immunogenicity. Development of polysaccharide glycoconjugate vaccines has dramatically decreased the rates of invasive childhood diseases against *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, and *Neisseria meningitidis*. However, the lack of predictive animal models has made the development of novel conjugate vaccines difficult. Decisions regarding conjugation chemistries employed, selection of protein carriers used, and the size of polysaccharides have often times been discovered empirically from clinical data. Presented are several different methods for conjugation of *N. meningitidis*, and the resulting immune responses.

281: Characterization of a recombinant soluble form of the β-glucan binding domain of the human complement receptor type 3 (CR3).

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Complement receptor type 3 (CR3 or CD11b/CD18 or MAC-1) binds complement iC3b and stimulates the phagocytosis of iC3b opsonised pathogens and tumor cells. CR3 is a member of the β2 family of integrins and is expressed on the surface of neutrophils, macrophages and mature or activated NK cells. Polysaccharides, especially β3-glucans from yeasts, fungi or plants, bind to the receptor and induce a conformational change which activates the receptor by exposing the iC3b binding region. The glucans bind to the receptor via its lectin domain which is still poorly characterized. At present, its exact location in the CR3 extracellular domain as well as its carbohydrate binding specificity has not yet been determined with precision. To study in more detail the interaction between the lectin domain of the receptor and β-glucans we produced as recombinant proteins three soluble fragments of the extracellular domain which could contain the binding pocket for the β-glucans. Only fragments which had been mutated at one amino acid could be expressed successfully as secreted and soluble proteins. The proteins expressed and purified were characterized by SDS-PAGE and Western blotting. The binding activity towards β-glucans was tested by immobilizing the CR3 lectin domains and measuring their ability to retain fluorescent zymosan particles. All three recombinant proteins contain at least part of the glucan binding domain since all thre are able to bind zymosan. In conclusion, we have prepared soluble forms of the CR3 lectin domain and show that they are able to bind β-glucan particles. This is an important first step in the elucidation of the binding specificities of the CR3 lectin domain which will be important for the design of more efficient small activators of the complement receptor type 3.

282: Clec11a – C-type lectin family member in pathogen recognition

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Glycoreceptors (human lectins) are ligands of complex oligosaccharid structures of cell surfaces. Macrophages, dendritic cells, T-cells, NK cells as well as lymph and blood endothelials contains a variety of glycoreceptors. As receptors they bind to pathogens like bacteria, viruses, protozoons and parasites. Recent results gave evidence that glycoreceptores influences the progression of cancer. Glycans of the cell surface of invasive cancer cells bind to cellular glycoreceptors. From human genome several grups of glycoreceptors are known, C-type lectins, galectins, siglecs, however, it is a challenge to get the active lectin from cell culture. The present work focuses on the cell line development, expression and purification of C-type lectins. The expression was performed in HEK cells and converted for up-scale in hybridoma media. Purification was performed with supported media functionalized with glycans with special emphasis on the Calcium dependence of the C-type lectins. For the first time binding studies of C-type family member Clec11a on carbohydrate micro assays were presented.

283: Body fluids: sweet protection against infection?

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We show that the human innate immune system has evolved to use the infection mechanism of pathogens to its own advantage by using the same protein-sugar interactions to bind the pathogen to glycoproteins in various human biological fluids. Once bound, the pathogens can then be expelled physically from the body.

In particular, using carbon LC-ESI-MS/MS, we show that breast milk has a different oligosaccharide composition to cow's milk and that the sugar epitopes exposed on proteins in human breast milk serve to bind specific bacterial pathogens that might otherwise infect the infant gut. A similar phenomenon occurs in eyes in which the tear film of the eye protects this crucial exposed surface from pathogen infection. The eye has a complex tear film comprising glycoproteins which display specific sugar epitopes to the environment and the structures of these provide a protective mechanism against the bacterial species known to infect the eye. In addition, buccal epithelial cells lining the oral cavity are sites of interaction of oral pathogens. These interactions involve adherence between the glycans of the host cells and the carbohydrate binding proteins of the pathogen. We show that there is an innate immune mechanism provided by the glycoproteins in the flow of saliva that constantly bathes the epithelial surface of the mouth, which competes with pathogen adhesion to the cells. Exploration of the molecular mechanisms operating in the protection of the epithelial surfaces by the secreted body fluids opens up the possibility of new preventative therapies using molecular mimics.

284: Glycomics of human breast milk: an antimicrobial defence mechanism

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Human milk contains a highly potent mixture of protective agents such as free sugars, antibodies and anti-microbial proteins constituting an innate immune system, whereby the mother protects her infant from enteric and other diseases. Human milk free sugars have been reported to inhibit microbial pathogenesis in various in vivo, ex vivo and in vitro experimental systems however the changes which occur to specific sugar structures attached to glycoproteins in milk over the course of lactation have not been investigated. Glycoproteins are contained in the soluble fraction of milk as well as in the milk fat globule membrane (MFGM) which surrounds and solubilises the lipid. The oligosaccharide structures expressed on these proteins were found to be blood group independent of the individual, so that it is possible that the protection offered by these sugars in milk is common to all women. The N- and O-linked glycan phenotype, both of the total glycoprotein complement as well as of specific glycoproteins, of milk from five different individuals during the course of infant breastfeeding will be presented. The structures of those protein-attached glycans which are recognized by human gut pathogens will be described. These oligosaccharide moieties present on the milk glycoproteins, with homology to epithelial mucus cell surface pathogen receptors in the stomach and intestine, may inhibit infection by competitively binding with the pathogens and clearing them from the infant gut. This knowledge offers the opportunity to design glycoconjugates that may be added to infant milk formula to help protect infants from endemic pathogens.

Poster Session I (Monday)

Physiology & Signalling

285: Activation of monohexosylceramide synthesis under heat stress contributes to down-regulate the intracellular ceramide level as a ceramide scavenger and modified more complex sphingoglycolipids metabolism

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Heat stress on MDCK cells increased ceramide content to 187% at 40°C for 24 h, and the de novo synthesis from serine increased to 146%. Glucosylceramide (GlcCer) and galactosylceramide (GalCer) synthesis from ceramide increased to 290% (GalCer) and 143% (GlcCer) after metabolic labeling with ^{14}C-glucose at 42°C. Lactosylceramide also increased to 151%, whereas sulfatide (SM4s) and ganglioside GM3 decreased to 21% and 43%, respectively. SM4s showed optimal sulfation at 37°C, whereas cholesterol sulfate was optimally sulfated at 40°C. The gene expression of ceramide glucosyltransferase (*GlcT*), ceramide galactosyltransferase (*GalT*), and GalCer sulfotransferase (*GST*) after 24 h culture at 42°C significantly increased to 714%, 221%, and 174%, respectively. Although *GST* gene expression was higher under heat stress, SM4s synthesis decreased, which may have been due to increased GST sensitivity to a temperature higher than 37°C. When we introduced the HSP70 gene into the expression vector and transfected the plasmid (pCDM-dHSP70) into kidney cells, GlcCer synthesis increased significantly. HSP70 may play a role in GlcT gene expression to increase GlcCer and decrease intracellular ceramide level. We investigated ceramide accumulation in mouse fibroblast Mop 8 cells and effects on ceramide metabolism-related genes, i.e., a serine palmitoyltransferase (*Spt*), ceramide synthase 1 and 2 (*CerS1* and *CerS2*), a sphingomyelin synthase 1 (*SgmS1*), neutral sphingomyelinase 1 and 2 (*nSMase1* and *nSMase2*), and a ceramide glucosyltransferase (*GlcT*) under conditions of heat stress. All of these genes were activated under heat stress at 42°C. Activation of *Spt*, *CerS1*, *CerS2*, *nSMase1*, and *nSMase2* genes contributed to the increase in ceramide

levels, whereas *SgmS1* and *GlcT* genes, as ceramide scavengers, played a role in the decrease of intracellular ceramide levels. The transformant cell (MopGT), which was selected as a strain stably expressing GalCer, showed a novel expression of GalCer and a significant decrease in intracellular ceramide levels. These results suggested that ceramide glycosylation contributes to regulate the intracellular ceramide levels.

286: Sulfoglycolipid synthesis in mammalian renal cells is regulated under osmotic stress by the osmolarity of medium via MAPK signaling pathway

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Sulfatide (SM4s) in MDCK cells increased in a hypertonic medium supplemented with sodium chloride. TLC showed that hypertonic stress induced the accumulation of GalCer as a precursor of SM4s. The level of ceramide as a precursor of both GalCer and GlcCer increased under hypertonic stress and decreased under hypotonic stress. GalCer sulfotransferase (GST) mRNA was shown to be elevated in the hyperosmotic condition. The increase in SM4s under hypertonic stress was induced by the activation of both the galactosyltransferase (GalT) and GST genes. Verots S3 and Vero317 cells were shown by metabolic labeling with ^{35}S-sulfate to contain many more sulfoglycosphingolipids than original Vero cells. The activity of GST was shown to be 89- and 92-fold higher in Vero317 cells and Verots S3 cells, respectively, than that of the parent cells, whereas the activity of the degradation enzyme, arylsulfatase A, was unchanged among all the three cell strains. GST gene transcript levels in Verots cells were 14.3-fold higher than those in Vero cells. The cell adhesiveness to the culture plate under hypertonic stress was strengthened significantly in both mutant strains. Among the major sulfoglycolipids of the Verots S3 cell line, the incorporation of ^{35}S-sulfate into SM3, SM2a and SB1a was upregulated with the increasing tonicity of the medium. Sulfoglycolipids in these renal cells seemed to contribute to the membrane barrier against hypertonic media. Sulfoglycolipid synthesis was suppressed with the p38 (MAPK) inhibitor SB203580 and/or with the MEK-1/2 (MAPKK) inhibitor PD98059, and with the tyrosine kinase inhibitor genistein, which also reduced the sulfoglycolipid synthesis in a dose-dependent manner. Further the administration of the MAPK/MAPKK inhibitors to the culture medium reduced significantly the viability of Verots S3 cells under hypertonic stress. These findings suggest that sulfoglycolipid synthesis in renal cells may be regulated to adapt to the renal osmotic circumstances by the medium's osmolarity via the MAPK signaling pathway.

287: Anti-proliferative activity of fucoidans from a brown seaweed (*Laminaria angustata* var. longissima) in U937 cells

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We have isolated and characterized fucoidans from a brown seaweed (*Laminaria angustata* var. *longissima*), which has been eaten in large quantity for 300 years in Okinawa, Japan[1,2]. The fucoidans consisted of four fractions, the main fraction of which was (1→3)-linked α-L-fucopyranosyl residues, a part of which had (1→2)-α-L-fucopyranosyl residue at the C-2 position on the main chain, and sulfate group at C-4. The fucoidans induced macrophage activation [2]. We report herein anti-proliferative activity of the fucoidans. Fucoidans were prepared as reported previously [1,2]. U937 cells, which is human leukemia cell line, was obtained from Human Sciences Research Resources Bank, Tokyo, Japan (JCRB9301). To examine whether the anti-proliferative activity of the fucoidans was caused by induction of apoptosis, apoptosis assay, caspase-3 and -7 activity assay, and Western blotting analysis were performed. The results of morphological analysis and APOPercentage apoptosis assay showed that the fucoidans induced apoptosis in U937 cells. The full-lingth 116 KDa PARP as a target of caspase-3 and -7 was cleaved to 35, 19 and 17 KDa fragments. The result indicated that the fucoidans exhibited anti-proliferative effect by inducing apoptosis associated with degradation PARP, caspase-3 and -7 activation in U937 cells. *L.angustata* var. longissima is a major traditional foodstuff because it has been eaten for 300 years in Okinawa, Japan. In recent years, the Okinawa population has the best health and greatest longevity on the planet. The kelp perhaps contributes to a healthy life and longevity in the Okinawa population.

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288: *Silurus asotus* lectin induces dormant status in Burkitt's lymphoma Raji cells

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Rhamnose-binding lectin isolated from eggs of catfish (*Silurus asotus*), SAL, is a 32 kDa protein and consists three tandem repeat domains of 95 amino acid residues. By the observation of direct interaction between SAL and

tumor cells, we found that SAL bound to Gb3-expressing Raji cells but not to Gb3-negative K562 cells. On the other hand, surface plasmon resonance spectrometric analysis revealed that the affinity constant of SAL for Gb3 was nearly equivalent to that for Galα1-XGal-BSA (X: 2, 3 and 4). Using TLC blotting and Western blotting, we also demonstrated that SAL bound to Gb3 in glycosphingolipid-enriched microdomain (GEM) prepared from Raji cells, and to glycoproteins in soluble high-density fractions prepared from not only Raji cells but also K562 cells. These results suggest that SAL binding to Gb3 may prevent SAL binding to glycoproteins possessing Galα-linked carbohydrate chains. Consequently, fluorescence of SAL may be observed in the surrounding of Gb3-expressing cells but not Gb3-negative cells. It may be characteristic of the Gb3 molecule in GEM to cause SAL-mediated signals, as follows. SAL bound to Gb3 on Raji cells, and led the cells to early stage apoptosis. SAL binding to Gb3 caused 1) externalization of phosphatidylserine via activation of multidrug resistance 1 P-glycoprotein, 2) cell volume decrease, following efflux of potassium ion via activation of Kv1.3, 3) cell cycle G1 arrest via down-regulation of cyclin-dependent protein kinase 4, and up-regulation of p21 and p27, and 4) inhibition of cell proliferation, but 5) did not cause activation of caspase and induction of DNA fragmentation and apoptotic nuclei. That is, SAL induces early apoptotic status, but not the switch to late apoptotic status in Raji cells. After withdrawal of SAL from the culture medium, SAL-induced phenomena were released in Raji cells in SAL-free medium. Since inactivation of glutamate dehydrogenese and reduction of expression of isocitrate dehydrogenese were observed in SAL-treated Raji cells, it seems that SAL induces dormant status via regulation of citric acid cycle.

289: MUC1 mediated signaling through ligation with Siglec 9

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Membrane-bound mucins have been considered to play important biological roles in cell to cell and cell to matrix interactions. Recently, it is reported that they also function as an intracellular signaling molecule which is responsible for malignant phenotype. MUC1 is a heavily glycosylated membrane-bound mucin and is translated as a single polypeptide, which is cleaved in the endoplasmic reticulum, yielding N- and C-terminal subunits that form a heterodimeric complex, bound by non-covalent interactions. An extracellular binding protein responsible for the signaling through MUC1 itself has not been extensively studied. In many carcinomas, infiltrating immune cells are found closely associated with tumor cells but little is known concerning the nature or significance of adhesion and/or recognition molecules involved in this cellular interactions To assess MUC1-mediated signaling, we used mouse 3T3 cells transfected with MUC1cDNA rather than epithelial cancer cell lines in order to more clarify the function of MUC1 in cells lacking tumor associated genetic and epigenetic changes. In some epithelial cancer tissues, we found immunochemically that MUC1 expressing cancer cells associated with infiltrated immune cells expressing Siglec-9. MUC1 was detected in the proteins pull-downed from MUC1/3T3 cell lysate by recombinant Siglec-9. Binding of Siglec-9 to MUC1 was also confirmed by a plate assay using purified MUC1 and recombinant Siglec-9. When MUC1/3T3 cells were incubated with recombinant Siglec-9, phosphorylation of ERK1/2 and recruitment of β-catenin to MUC1 were elevated in a dose dependent manner. In contrast, phosphorylation of β-catenin was decreased and the treatment with pp2 (Src inhibitor) decreased the recruitment of β-catenin. Accumulation of β-catenine in a nuclei was also observed immunochemically and confirmed biochemically in Siglec-9 treated MUC1/3T3 cells. Growth of MUC/3T3 cells was elevated by treatment with Siglec-9. These results suggest that MUC1 mediated signaling through ligation with Siglec 9 plays a role in tumor-progression.

290: Lectin-related degradation of glycoproteins in the nucleus and cytoplasm of plant cells

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Protein degradation is widely used in eukaryotes to control the abundance of key proteins and the removal of abnormal proteins. A conserved proteolytic pathway involves the endoplasmic reticulum associated degradation system, better known as the ERAD pathway. In this quality control system improperly folded proteins or incompletely assembled oligomers are retained in the ER and translocated to the cytosol where they are degraded by the ubiquitin-26S proteasome system. A crucial step in this pathway – the selective recognition of target proteins – is mediated by F-box proteins. The F-box protein family is the largest protein superfamily known, the total number of members ranging from 20 in yeast and 70 in humans to more than 700 proteins in plants. Substrate binding of F-box proteins relies mostly on protein-protein interactions. Recent studies in mammals have documented the existence of a group of F-box proteins (Fbs proteins) that have a lectin domain which is involved in recognition and binding of glycoproteins that fail to achieve proper folding or assembly. After binding of these Fbs proteins with a misfolded glycoprotein, the glycoprotein is labeled with ubiquitin and degraded by the proteasome. Unfortunately, very little information is available on the degradation of misfolded or incompletely assembled glycoproteins in plants. Through extensive searches in plant genome databases, our group was able to identify a small number

of putative plant-specific Fbs proteins. These F-box proteins contain a C-terminal domain that is homologous to Nictaba, a nucleocytoplasmic lectin from tobacco plants. This lectin exhibits specificity towards high-mannose N-glycans and can interact with many plant (glyco)proteins. Based on striking similarities with the mammalian Fbs proteins, it is hypothesized that the putative plant Fbs proteins fulfill a function in glycoprotein turnover similar to the mammalian Fbs proteins.

291: Annexin A5 regulates blood coagulation via its carbohydrate recognition domains.

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The annexins are a family of calcium-dependent membrane-binding proteins. Twelve human annexins have been identified and shown to be involved in a diverse range of physiological and pathological processes. Membrane phospholipids such as phosphatidylserine are well-studied ligands for annexins. However, a previous study in our laboratory demonstrated that several mammalian annexins including annexin A5 (anxA5) specifically bind to glycosaminoglycans (*J. Biol. Chem.*, 273 (1998) 9935-9941). Moreover X-ray crystallography demonstrated that anxA5 has two carbohydrate recognition domains (*Structure* 9 (2001) 57-64). One well-known physiological activity of anxA5 is anticoagulation, and ANXA5 gene variants affect the risk for recurrent pregnancy loss due to hypercoagulation disorders (*J. Autoimmun.*, 15 (2000) 107-111). Proposed mechanisms for the expression of anxA5 anticoagulant activity are sequestration of phosphatidylserine and inhibition of the steps activated by phosphatidylserine during the blood coagulation cascade. However, it is not clear whether carbohydrate binding activity is involved in the expression of anxA5 anticoagulant activity.

In this study, we prepared recombinant human anxA5 and its mutants by substituting amino acid residues responsible for interaction with heparin tetrasaccharide, and compared their phospholipid or glycosaminoglycan binding and anticoagulant activities. In contrast to uniform phospholipid binding activities, the glycosaminoglycan binding activities were distinctive. All anxA5 mutants that exhibited reduced glycosaminoglycan binding activities had decreased anticoagulant activities. Furthermore, heparan sulfate inhibited the anticoagulant activity of anxA5. These results revealed that anxA5 regulates its anticoagulant activity by binding to glycosaminoglycans.

To confirm that anxA5 binds to cell surface glycosaminoglycans, we performed cell fluorescence analyses of CHO-K1 and CHO-*pgs*745, which is a glycosaminoglycan-deficient cell line. The fluorescence intensity of anxA5 reacted with CHO-K1 was higher than with CHO-*pgs*745, and it was decreased by inhibition of heparin. These results showed that anxA5 binds to cell surface glycosaminoglycans via its carbohydrate recognition domains, suggesting that anxA5 may regulate blood coagulation by interacting with proteoglycans on the cell surface of endothelial cells.

292: Role of N-glycosylation for the activity of the *Arabidopsis thaliana* endo-1,4-beta-glucanase KORRIGAN1

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A major challenge of plant glycobiology is the characterization of the biological functions of N-linked oligosaccharides. Although all enzymes of the plant N-glycosylation pathway have been characterized at large and mutants with defined N-glycosylation patterns are available, the elucidation of the physiological function of N-glycans in the Golgi and beyond lags behind. We have recently reported that changes in N-glycosylation result in pronounced effects on cell wall formation in *Arabidopsis thaliana* (Plant Cell (2009) 21: 3850-3867). This has been proposed to be due to altered glycosylation of proteins involved in cell wall synthesis. One potential target glycoprotein is the plasma membrane-bound endo-1,4-β-glucanase KORRIGAN1 (KOR1) which is essential for plant cell expansion and cellulose synthesis. Specific genetic interactions between a weak kor1 allele and a number of different Arabidopsis N-glycan processing mutants indicate that maturation of complex N-glycans is crucial for full functionality of KOR1, a glycoprotein harbouring 8 N-glycosylation sites in its catalytic domain. To investigate the functional significance of proper KOR1 N-glycosylation in more detail, we have now expressed a soluble variant of the enzyme in insect cells. Interestingly, the activity of recombinant KOR1 was found to be substantially reduced upon enzymatic deglycosylation with PNGase F. To further characterize the impact of N-glycosylation on KOR1 activity, all potential N-glycosylation sites were individually eliminated by site-directed mutagenesis. The effect of these mutations on the enzymatic activity of recombinant KOR1 was then analyzed by endo-1,4-β-glucanase activity assays using carboxy-methyl cellulose as substrate. In addition, the glycan structures present on each N-glycosylation site of recombinant KOR1 were determined by LC-ESI-MS analysis of tryptic glycopeptides. Our data show that the presence of complex N-glycans on selected N-glycosylation sites is critical for KOR1 enzyme activity.

293: Molecular mechanisms for the growth disorders in GM3-only mice

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Glycosphingolipids (GSLs) are localized in membrane microdomains and are involved in the regulation of membrane protein functions and signal transduction. Small intestinal epithelial cells are enriched with characteristic GSLs, and it is reported that the main GSL compositions change from sialylated GSLs into asialo-GM1 during postnatal development. Mice lacking the GM2/GD2 synthase and the GD3 synthase genes (GM3-only mice) were born almost normally at a glance, though they soon showed growth disorders and sudden death that seemed to be derived from malnutrition. To elucidate the molecular mechanisms for the growth disorders due to the deficiency of GSLs, we investigated changes in morphology and expression profiles of nutrient transporters in the small intestine of the GM3-only mice. After the weaning, the GM3-only mice showed disarrangement of enterocytes lining along villus and shorter villi compared with wild type mice. Quantitative real-time RT-PCR revealed the down-regulations of some nutrient transporters including SGLT1, FATP4, FABP2, PEPT1, rBAT, 4F2hc, and B(0) AT1 in jejunum of the GM3-only mice at 4 weeks after birth, while there were no significant differences in the expression levels of these transporters during the suckling between the GM3-only and wild type mice. These results suggest that abnormal GSL composition, including asialo-GM1 deficiency after weaning, result in the reduced nutrient absorption via dysfunctions of transporters. To clarify the molecular mechanisms for the absorption disorders in the GM3-only mice, detailed analyses on the functions of these transporters with focus on relationship with GSLs are now under investigation.

294: Physiopathological function of GM3 ganglioside

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Since I was involved in the molecular cloning of GM3 synthase (SAT-I), which is the primary enzyme for the biosynthesis of gangliosides in 1998, my research group has been concentrating on our efforts to explore the physiological and pathological implications of gangliosides especially for GM3. During the course of study, we demonstrated the molecular pathogenesis of type 2 diabetes and insulin resistance focusing on the interaction between insulin receptor and gangliosides in membrane microdomains and propose a new concept: Life style-related diseases, such as type 2 diabetes, are a membrane microdomain disorder caused by aberrant expression of gangliosides. We also encountered an another interesting aspect indicating the indispensable role of gangliosides in auditory system. After careful behavioral examinations of SAT-I knockout mice, their hearing ability was seriously impaired with selective degeneration of the stereocilia of hair cells in the organ of Corti. This is the first observation demonstrating a direct link between gangliosides and hearing functions.

295: Platelet is involved in B-CLL progression through CLEC-2/CD74 interaction

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CLEC-2 is a type II transmembrane C-type lectin-like receptor, and readily detected on platelet and megakaryocyte. In human platelet, CLEC-2 is detected as a doublet by western blot, with a major band migrating at 32kD and a minor band at 40kD. Recent studies demonstrate that CLEC-2 recognizes snake venom rhodocytin and podoplanin. In addition, CLEC-2 also co-operates with DC-SIGN to facilitate the capture of HIV-1 by platelets. In this study, we perform a yeast two-hybrid screening in human leukemia cDNA library to explore the potential endogenous ligand of CLEC-2 and gain insight into its biological function. By using the extracellular region of human CLEC-2 (hCLEC-2) as bait, we identify CD74 as a potential ligand for CLEC-2 in yeast. Recent studies reveal that CD74 is highly expressed on the surface and mediates the proliferation of B chronic lymphocyte leukemia (B-CLL) cells. By expressing and purifying the recombinant protein of the extracellular part of CD74, we identify in vitro that CD74 selectively recognizes the 40kD form of hCLEC-2, but not its 32kD form. Confocal and FACS analysis also confirm that CD74 recombinant protein binds to CLEC-2 on cell surface. Further research reveals that administration of soluble CLEC-2 promotes the activation of ERK, AKT and STAT3 signaling pathways in primary B-CLL cells through CD74, leading to cell proliferation and chemo-resistance. Co-culturing with platelet under static and flowing conditions also promotes the proliferation of B-CLL cells in CLEC-2 and CD74 dependent manner. These results indicate that the 40kD form of CLEC-2 recognizes CD74 and promotes the initiation of its downstream signaling, and suggest that platelet is involved in the progression and development of B-CLL through CLEC-2-CD74 interaction, thus providing CLEC-2 as a potential target for the treatment of B-CLL patients.

296: Hijacking a biosynthetic pathway yields a glycosyltransferase inhibitor within cells

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The addition and removal of a GlcNAc moiety to serine or threonine residues of proteins (O-GlcNAc) is a common post-translational modification in multicellular eukaryotes. It occurs on hundreds of proteins in vivo, and is sometimes reciprocal with phosphorylation. Two enzymes are responsible for maintaining O-GlcNAc levels on proteins; a glycoside hydrolase (OGA) removes the sugar from a protein, and a glycosyltransferase (OGT) installs the O-GlcNAc moiety. The molecular basis for the function of O-GlcNAc in both health and disease is being decoded using genetic and chemical tools. Inhibitors of OGA have been described and are proving useful for studying O-GlcNAc in cells. Inhibitors of OGT, however, are lagging behind and few compounds have been well characterized as acting effectively in cells to decrease O-GlcNAc levels. Indeed, despite great interest, the development of glycosyltransferase inhibitors in general is a difficult problem. Recently, we have developed a small molecule compound that, when administered to cells, reversibly decreases O-GlcNAc levels in a dose and time dependent manner. This compound works in an unusual manner. The compound can enter cells and hijack the cell's own biosynthetic machinery in order to create a mimic of the natural substrate donor, which acts as an effective inhibitor of OGT[1]. We anticipate that this will be a useful tool to study the effects of decreasing the O-GlcNAc modification in cells and in vivo. In addition, by adaptation, inhibitors of different classes of glycosyltransferase may now be accessible. 1) Gloster, T. M., Zandberg, W. F., Heinonen, J. E., Shen, D. L., Deng, L. and Vocadlo, D. J. (2011), Nat Chem Biol, 7, 174-181.

Poster Session II (Thursday)

Biosynthesis & Metabolism of Glycoconjugates

297: Sugar nucleotide analysis by ESI-MSMS using porous graphitic carbon

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Nucleotides and nucleotide sugars are pivotal components of metabolic and anabolic pathways of cells. Their polar nature and chemical lability as well as the occurrence of isobaric structures render their determination a difficult task. Here we present the analysis of a wide range of nucleotide sugars and nucleotides by chromatography on a reduced porous graphitic carbon column in capillary format with mass spectrometric detection using a fully volatile buffer without ion pairing reagents. A rapid sample preparation procedure was developed that allowed handling and detection of the very short-lived bacterial CMP-2-keto-desoxy-octulosonic acid in *E. coli*. The chromatography revealed unexpected isomers of cyclo-, mono-, di and triphosphates of various nucleotides. Rarely considered nucleotide sugars such as UDP-L-arabinopyranose, UDP-L-arabinofuranose, GDP-L-galactofuranose, UDP-L-rhamnose, GDP-sulfoquinovose, ADP-ribose as well as ADP-glucose were detected in plant extracts. Corresponding peaks for UDP-Xyl as well as UDP-Ara*p* were found in CHO-cells. The labile CMP-Neu5Ac as well as CMP-Neu5Gc were found in liver extracts from mouse. dTDP-Rha and several GDP-hexose isobars could be found in various bacterial cells. The overall time consumption from harvesting the plants (1-10 mg of fresh weight) or cells (10^5 CHO-cells) to the ready prepared samples for LC-ESI-MSMS analysis is approximately 1 hour.

Reference: Pabst et al. (2010) Anal Chem. 82(23): 9782–9788

298: Computational analysis and comparison of glycosphingolipids among invertebrates with newly constructed wiki-based LipidBank database

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LipidBank (http://lipidbank.jp), the official database of the Japanese Conference on the Biochemistry of Lipids (JCBL, http://jcbl.jp), is a publicly free database of natural lipids including fatty acids, glycerolipids, sphingolipids, steroids, and various vitamins. The database offers more than 7,000 unique molecular structures (ChemDraw and MOL format), their names (common and IUPAC names), spectral information (mass, UV, NMR and others), and most importantly literature information. This database is widely used in over 60 countries and there are many repeat users. LipidBank is collaborating with Lipid MAPS consortium (http://www.lipidmaps.org/) in U.S.A., European Lipidomics Initiative consortium (http://www.lipidomics.net/) and LipidomicNet (http://www.lipidomicnet.org/) to establish lipid nomenclatures and lipidomics. To modernize LipidBank which became open in 1999, we are now changing operating system using

MediaWiki, a free software that enables us easily to register the new data, link to other websites, update and keep a record of changes made to wiki pages. In July 2009, the lipid class, fatty acyl, was renewed using wiki system (http://lipidbank.jp/wiki/Category:LBF). In this presentation we report about the glycosphingolipids among the invertebrates. Glycosphingolipids play important roles in membrane structure and function, signal transduction, cell regulation, and diseases such as neurodegeneration, diabetes, and cancer. 581 registered data of glycosphingolipids in LipidBank have been converted to a wiki-based system. Also, we recently collected 405 glycosphingolipids-and-species relationships for invertebrates from Ref. 1. Computational analysis of genera-specific characters of glycosphingolipids, such as comparison of sugar-chains and linkage types will be useful for taxonomic classification concurrently with rRNA-based phylogeny.

[1] S. Itonori, M. Sugita "Glycophylogenetic Aspects of Lower Animals" Chapter 3.15, pp.253-284 In: Johannis P. Kamerling (Ed.) Comprehensive Glycoscience: From Chemistry to Systems Biology, Elsevier 2007

299: Selective Modulation of Glycosphingolipid Metabolism by Soluble Glycosphingolipid Analogues

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Mammalian glycosphingolipid (GSL) precursor monohexosyl ceramides are either glucosyl or galactosyl ceramide (GlcCer, GalCer). Most GSLs derive from GlcCer. We have created analogues of GSLs by substituting the GSL fatty acid with adamantane, generating amphipathic mimics of increased water solubility, while retaining receptor function. We have synthesized adamantylGlcCer and adamantylGalCer, which partition into cells to alter GSL metabolism. At low dose, adaGlcCer increased cellular GSLs by inhibition of glucocerebrosidase (GCC). Recombinant GCC was inhibited at pH 7 but not 5. In contrast, adaGalCer stimulated GCC at pH 5 but not 7, corrected N370S mutant GCC traffic from ER to lysosomes and reduced GlcCer accumulation in Gaucher disease cells, the most common of the lysosomal storage diseases (LSDs). At 40 μM adaGlcCer, lactosyl ceramide (LacCer) synthase inhibition depleted LacCer (and more complex GSLs), such that only GlcCer remained. In Vero cell microsomes, 40 μM adaGlcCer was converted to adaLacCer and LacCer synthesis inhibited. AdaGlcCer is the first LacCer synthase inhibitor. At 40 μM adaGalCer, cell synthesis of only Gb_3 and Gb_4 was significantly reduced and a novel product, adamantyl digalactosyl ceramide ($adaGb_2$) generated, indicating substrate competition for Gb_3 synthase. Microsomal Gb_3 synthesis was inhibited by adaGalCer. Metabolic labeling of Gb_3 in Fabry disease cells was selectively reduced by adaGalCer and $adaGb_2$ produced. $AdaGb_2$ in cells was 10-fold more effectively shed into the medium than the more polar Gb_3, providing an easily eliminated 'safety valve' alternative to Gb_3 accumulation. Adamantyl monohexosyl ceramides thus provide new tools to selectively manipulate normal cellular GSL metabolism and prevent GSL accumulation in cells from LSD patients.

300: Chondroitin 4-O-sulfotransferase-1 regulates the chain length of chondroitin sulfate in cooperation with chondroitin N-acetylgalactosaminyltransferase-2

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Previously, we demonstrated that sog9 cells, a murine L cell mutant, are deficient in the expression of chondroitin 4-O-sulfotransferase-1 (C4ST-1) and that they synthesize fewer and shorter chondroitin sulfate (CS) chains. These results suggested that C4ST-1 regulates not only 4-O-sulfation of CS but also the length and amount of CS chains; however, the mechanism remains unclear. Here, we demonstrated that C4ST-1 regulated the chain length and amount of CS in cooperation with chondroitin N-acetylgalactosaminyltransferase 2 (ChGn-2). Overexpression of ChGn-2 increased the length and amount of CS chains in L cells, but not in sog9 mutant cells. Knockdown of ChGn-2 resulted in a decrease in the amount of CS in L cells in a manner proportional to ChGn-2 expression levels, while the introduction of mutated C4ST-1 or ChGn-2 lacking enzyme activity failed to increase the amount of CS. Furthermore, the non-reducing terminal 4-O-sulfation of N-acetylgalactosamine residues facilitated the elongation of CS chains by chondroitin polymerase consisting of chondroitin synthase-1 and chondroitin polymerizing factor. Overall, these results suggest that the chain length of CS was regulated by C4ST-1 and ChGn-2 and that the enzymatic activities of these proteins played a critical role in CS elongation.

301: Distinct roles of b1,3-N-acetylglucosaminyltransferases and carbohydrate 6-O sulfotransferases on corneal keratan sulfate production

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Keratan sulfate glycosaminoglycan (KS-GAG) is mainly found in the cornea, and suggested to have an important role for maintenance of corneal transparency. KS-GAG

chains are linearly elongated disaccharide repeats of -3Gal β1-4GlcNAc β1- with sulfates on 6-O position of GlcNAc and Gal. We have been studying on biosynthesis of KS-GAG and found that four Golgi-localized enzymes, corneal GlcNAc 6-O sulfotransferase (CGn6ST, also known as GlcNAc6ST-5/GST4b), keratan sulfate Gal 6-O sulfotransferase (KSG6ST), β1,3-N-acetylglucosaminyltransferase-7 (β3GnT7) and b1,4 galactosyltransferase-4 (β4GalT4) are required for highly sulfated KS-GAG production in vitro and in vivo. Using sulfotransferase-expressing HeLa cells, we further analyzed involvement of other β1,3-N-acetylglucosaminyltrasnferases for KS-GAG production and found that β1,3-N-acetylglucosaminyltransferase-2 (β3GnT2) has significant activity for KS-GAG production in the cells. We also investigated the role of KSG6ST for highly sulfated KS-GAG synthesis in vivo using gene knockout mice and found that KSG6ST is required for highly sulfated KS-GAG production in the cornea, as in the case with CGn6ST. However, unlike heterozygotes of CGn6ST-deficient mice, the amount of highly sulfated KS-GAG is largely decreased in the cornea of heterozygous KSG6ST-deficient mice. This result confirms the hypothesis that sulfation on Gal occurs after production of GlcNAc-sulfated polylactosamine chains, and degree of Gal-sulfation in the GAG chains corresponds to the amount of KSG6ST enzyme.

302: Functional enzyme complex of β-1,4-galactosyltransferase-II and GlcAT-P in HNK-1 biosynthesis

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HNK-1 carbohydrate is characteristically expressed on a series of cell adhesion molecules in the nervous system. The expression is spatially and temporally regulated during the development of the nervous system. The HNK-1 carbohydrate has a unique structure comprising a sulfated trisaccharide (HSO_3-3GlcAβ1-3Galβ1-4GlcNAc-) and is biosynthesized by the sequential reactions of beta-1, 4-galactosyltransferase (β4GalT), glucuronyltransferase (GlcAT-P, GlcAT-S), and sulfotransferase (HNK-1ST). Previous study showed that mice lacking β4GalT-II, one of seven β4GalTs, exhibited a remarkable reduction in HNK-1 expression. However, HNK-1 expression was unaltered in the β4GalT-I-deficient mouse brain despite the similarity in primary structure and acceptor specificity of these two enzymes, indicating that β4GalT-II has specific roles in the biosynthesis of HNK-1 carbohydrate. Here we investigated the underlying molecular mechanism of the regulation of HNK-1 expression. First, by performing a lectin blot analysis we revealed that N-acetyllactosamine, which is the inner structure of HNK-1, remains almost equally in the whole brain of β4GalT-II-deficient mice as compared to wild-type. This result indicates that the disappearance of HNK-1 carbohydrate observed in β4GalT-II-deficient mice is not likely due to a general loss of the β1, 4-galactose residue. Then, by co-immunoprecipitation and ER-retention analyses using Neuro2a cells, we demonstrated that β4GalT-II but not β4GalT-I physically and specifically associates with GlcAT-P. In addition, we revealed by pull-down assay that Golgi luminal domains of β4GalT-II and GlcAT-P are sufficient for formation of a hetero-enzyme complex. Finally, we showed that co-expression of β4GalT-II and GlcAT-P generally enhanced HNK-1 expression on various glycoproteins in Neuro2a cells including NCAM, suggesting a regulatory function for this complex in HNK-1 production in vivo.

303: Purification and characterization of plant β-Xylosidase involved in turnover of plant complex-type N-glycans

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β-Xyl'ase is one of essential glycosidases involved in the turnover of plant N-glycans as well as hemicellulose of plant cell wall. Although some genes of Arabidopsis β-Xyl'ases involved in secondary cell wall metabolism were identified, a β-Xyl'ase responsible for degradation of plant complex-type N-glycan (PCNG) remains to be identified and characterized. In this study, we purified a β-Xyl'ase (β-Xyl-Le1) from homogenates of tomato fruits by ion-exchange chromatography, hydrophobic chromatography and gel filtration chromatography. The molecular mass of the purified β-Xyl-Le1 was estimated to be 67 kDa by SDS-PAGE and 60 kDa by gel filtration, indicating the enzyme has a monomeric structure. The N-terminal amino acid was blocked and the optimum pH was 4.0 for pNP-β-xyloside and 5.0 for MFX (Man1Xyl1Fuc1GlcNAc2-PA), respectively. β-Xyl-Le1 could completely hydrolyze the β1-2 xylosyl residue from Manα1-6(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA (M2FX), MFX, and MX, but the Xylosidase was completely inactive against the typical PCNGs, M3FX or GN2M3FX, indicating clearly that the Manα1-3 arm affects the activity.

304: Molecular characterization and expression analysis of α-Fucosidase from tomato (*Solanum lycopersicum*)

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It has been revealed that metabolism of N-glycan is responsible for the plant growth or fruit maturation or ripening. Therefore, expression analysis of some glycoenzymes involved in N-glycan turnover is essential to understand the physiological functions of N-glycans linked to secreted or membrane glycoproteins in the fruit-ripening process. Plant α-Fucosidase (α-Fuc'ase) involved in degradation of plant complex-type N-glycan (PCNG) have not been characterised and the expression changes of the N-glycan-related α-Fuc'ase during fruit ripening process remains to be analyzed. In this study, therefore, we have cloned putative tomato α- Fuc'ases and analysed the changes in the expression levels in three tomato-ripening mutants (rin , Nr , alc) and two developing stages (green stage, red stage) by real time PCR. In tomato (*Solanum lycopersicum*) fruit, there are three putative α-Fuc'ase genes (Solyc03g006980, Solyc08g062280 and Solyc11g069010). It was found that the expression level of Solyc11g069010 was highest among these three putative α-Fuc'ase genes, and the gene expression level at green stage was much higher than that at red stage.

305: New families of TIM-barrel type hypothetical glycoside hydrolases

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Glycoside hydrolases or glycosidases [EC 3.2.1.-] are a widespread group of enzymes hydrolyzing the O-glycosidic bonds between two carbohydrates or between a carbohydrate and an aglycone moiety. All glycosidases are grouped into 120 families (GH1-GH125, except GH21, GH40, GH41, GH60, and GH69) in the CAZy classification. PSI-BLAST searches of the non-redundant database with several GH13, GH101, and GH114 TIM-barrel type catalytic domains as a query sequence allowed us to yield a large set of homologous proteins. Among them, using PSI Protein Classifier program, we selected proteins which do not belong to any known protein families. Based on homology they were grouped into fifteen families of hypothetical glycoside hydrolases: GHL1-GHL15. Domains of these families were used for iterative screening of the protein database by PSI-BLAST. Totally we revealed more than 26000 non-identical proteins. The majority of them contain domains belonging to several families of glycoside hydrolases: GH5, GH13, GH17, GH18, GH20, GH27, GH29, GH31, GH35, GH36, GH39, GH42, GH53, GH66, GH97, GH101, GH107, GH112, and GH114. Among others we found representatives of the following families: COG1082, COG1306, COG1649, COG2342, DUF3111, and PF00962. Some obtained proteins do not contain any known protein domains in the region homologous to GHL1-GHL15. Based on homology we grouped them into 34 new families of hypothetical glycoside hydrolases: GHL16-GHL49. Based on the sequence similarity we grouped some of the new families into superfamilies. One of them - the endo-α-N-acetylgalactosaminidase superfamily - includes families GHL1, GHL2, GHL3, GHL16, GHL18, GHL20, DUF3111, and GH101. The endo-α-1,4-polygalactosaminidase superfamily contains families GHL7, GHL8, COG2342, and GH114. The COG1649 superfamily groups families GHL4, GHL6, GHL9, GHL10, GHL11, GHL13, GHL30, GHL31, GHL48, GHL49, and COG1649. Families GHL5, GH13, GH70, and GH77 compose the α-glucosidase superfamily. We propose that 49 new families contain proteins with TIM-barrel type 3D structure and at least a few of the proteins have some glycosidase activities.

306: Changes of Substrate Specificity of Plant ENGase (Endo-Os) by Site-directed Mutagenesis

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Endo-ß-N-acetylglucosaminidase (ENGase) is involved in the significant production of high-mannose type free N-glycans during plant development or fruit maturation. In our previous reports, we identified genes of some plant ENGases (rice, tomato, and *Arabidopsis thaliana*) and constructed the gene-expression system. Furthermore, we reported that the knockout mutant (*Arabidopsis thaliana*), in which the two putative ENGase genes were knocked-out, produced the high-mannose type free N-glycans bearing the N,N'-acetylchitobiose (PNGase products) unit and showed no obvious morphological phenotype under standard growth conditions. As a par of study to reveal physiological significance of the plant ENGase involved in plant growth or fruit ripening, in this study, we identified the essential amino acids (N189, E191 and W236) for the hydrolytic activity by site-directed mutagenesis method. Furthermore, we succeeded to construct a mutant by site directed mutagenesis, which can hydrolyze a typical animal complex type N-glycans in addition to the high-mannose type ones.

307: The substrate recognition by human sialidase NEU2: a molecular modelling and site-directed mutagenesis approach.

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Sialidases or neuramidases are glycoside hydrolases removing terminal sialic acid residues from sialo-glycoproteins and sialo-glycolipids. Viral neuraminidases (NAs) have been extensively characterized and represent an excellent target for antiviral therapy through the synthesis of a series of competitive inhibitors that block the release of newly formed viral particles from infected cells. The human cytosolic sialidase NEU2 is the only mammalian enzyme structurally characterized and represents a valuable model to study the specificity of novel NA inhibitory drugs. Moreover, the availability of NEU2 3D structure represents a pivotal step toward the characterization of the molecular basis of the natural substrate recognition by the enzyme. In this perspective we have carried out a study of molecular docking of NEU2 active site using natural substrates of increasing complexity. Moreover, selective mutation of the residues putatively involved into substrate(s) interaction/recognition has been performed and the resulting mutant enzymes have been preliminary tested for their catalytic activity and substrate specificity. We found that E218 and Q270 are involved in the binding of the disaccharide α2,3-sialyl-galactose, whereas K45 and Q112 binds the distal glucose of the trisaccharide α2,3-sialyl-lactose, corresponding to the oligosaccharide moiety of the ganglioside GM3. In addition, D46 and also E218 were confirmed to be the key catalytic residues, supporting the catalytic mechanism hypothesized for these enzymes. Interestingly, the major effects detectable on the enzyme activity of NEU2 mutants are associated to residue(s) possibly involved in the recognition of the terminal sialic acid. In the near future this experimental approach will be used to study the recognition by NEU2 of more complex substrates, as well as the relative relevance of the linkage specificity, namely α2,3 versus α2,6 to galactose and α2,8 to a further sialic acid residue.

308: Double mutants as tools for elucidating N-glycosylation pathways in *Caenorhabditis elegans*

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C. elegans has a widespread utility in genetics and proteomics studies since it was first introduced by Sydney Brenner in 1963. Recent years, *C. elegans* began to be more popular in the study of glycomics. Interestingly, its glycome has been shown to have more complicated patterns than those of some higher organisms, e.g. Drosophila. The basis for this must be the presence of multiple glycosylation pathways involved in forming highly variable glycan structures. Here we report two novel double mutants as tools for elucidating N-glycosylation pathways in vivo: the H2H3 double mutant, lacking β-N-acetylhexosaminidase-2 (*hex-2*) and 3 (*hex-3*), and the F1F6 double mutant, lacking α1,3-fucosyltransferase-1 (*fut-1*) and 6 (*fut-6*). Preliminary data showed that the double mutants possess novel features. A loss of anti-HRP epitope (core α1,3-fucose) in an extract of the H2H3 double mutant, but not in the single *hex-2* and *hex-3* mutant extracts provides new evidence in biosynthesis of truncated N-glycans: HEX-2 and HEX-3 are main contributors in trimming the MGn to MM, together with Golgi mannosidase II, in the conversion of Man5Gn to MM. The abundance of a novel N-glycan structure (*m/z* 1646) in H2H3, with a Gal-Fuc modification on the inner GlcNAc of N-glycan core, increased the sensitivity to a toxic galectin CGL2. In comparison to the N2 wild type and *fut-1* or *fut-6* single mutant, the N-glycosylation pattern of the F1F6 double mutant has a general reduction in N-glycan fucosylation; no tetra-fucosylated glycan was detected whereas only a few tri-fucosylated glycans were apparent. Therefore, clues may be revealed when the glycan structures in each double mutant are carefully studied. It has to be noted that unexpected N-glycan structures were also observed in the double mutants; in particular, unusual N-glycan core modifications, which increase the complexity of any analyses.

309: Characterisation of the Class I α1,2-Mannosidases of *Caenorhabditis elegans*

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During the biosynthesis of N-glycans in multicellular eukaryotes, glycans with the compositions $Man_5GlcNAc_{2-3}$ are key intermediates. However, to reach this 'decision point', these N-glycans are first processed from $Glc_3Man_9GlcNAc_2$ through to $Man_5GlcNAc_2$ by a number of glycosidases, whereby up to four α1,2-linked mannose residues are removed by class I mannosidases (glycohydrolase family 47). Whereas in the yeast *Saccharomyces cerevisiae* there are maximally three members of this protein family, in higher organisms there are multiple class I mannosidases residing in the endoplasmic reticulum and Golgi apparatus. The genome of the model nematode *Caenorhabditis elegans* encodes seven members of this protein family, whereby four are predicted to be classical processing mannosidases and three are related proteins with roles in quality control. In this study, cDNAs encoding the four predicted mannosidases were cloned and expressed in *Pichia pastoris* and the activity of these enzymes, designated

MAS-1, MAS-2, MAS-3 and MAS-4, was verified. The first two can remove three residues from $Man_9GlcNAc_2$, whereas the action of the other two results in the appearance of the B isomer of $Man_8GlcNAc_2$; together the complementary activities of these enzymes result in processing to $Man_5GlcNAc_2$. With these data, another gap is closed in our understanding of the N-glycan biosynthesis pathway of the nematode worm.

310: Hunting an α-1,6-fucosyltransferase from the slug *Arion lusitanicus*

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Much is known in mammals about fucose in α 1,6-linkage to the asparagine-linked GlcNAc-residue of N-glycans and the corresponding glycosyltransferase catalyzing it – the α1,6-fucosyltransferase - but very little information is available for gastropods. In principle two approaches are possible for the identification and characterization of the α1,6-fucosyltransferase: construction of cDNA expression libraries and the direct biochemical purification. Full-length cDNA from *Arion lusitanicus* has been cloned first in *Pichia pastoris*. The library contained approximately $5x10^4$ colonies and sequencing showed no contamination with cDNA of any other organism. cDNA of the slug has also been cloned in the pBacPAK8 vector for recombination with the baculovirus genome to infect insect cells. First PCR screenings already showed colonies with different inserts, which increases the chance to find insect cells overexpressing recombinant α1,6-fucosyltransferase through screening for enhanced fucosylation with FACS. As it is not possible to use directly 2-aminopyridine (2-AP) labelled acceptor substrates for enzyme activity detection during screening and purification process, several more complicated detection systems had to be evaluated. Using both, the molecular as well as the biochemical approach, raises the chance to identify the gastropod α-1,6-fucosyltransferase and also other fucosyltransferases in snail tissues. This project is supported by the FWF project number P22118-B20.

311: An extraordinary N-glycan modifying enzyme in the animal kingdom, the ß1,2-xylosyltransferase from gastropods

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Xylose, ß1,2-linked to the ß-mannose of N-glycans, is a common sugar found in complex glycoconjugates of plants. Being absent in mammals, this modification constitutes an epitope for carbohydrate-reactive antibodies and is therefore responsible for allergic symptoms in humans. Gastropods, schistosomes and the ascidian Ciona intestinalis are so far found to be the only animals synthesizing ß1,2-xylosylated N-glycans. To study biochemical features of the responsible enzyme, our main goal is to purify and clone the ß1,2-xylosyltransferase from different snail species (*Arion lusitanicus*, *Achatina fulica* and *Biomphalaria glabrata*). Therefore, we constructed full-length cDNA libraries from *A. lusitanicus* in two different expression systems (the yeast *Pichia pastoris* and Sf9-insect cells). The level of gene diversity achieved in the libraries was estimated from PCR screenings and sequencing results. The cDNA library created in *P. pastoris* contained approximately 10^4 individual genes, whereas for the one in insect cells we calculated about $2x10^3$ single genes. These cDNA libraries were screened for enzyme activity (*P. pastoris*) or for ß1,2-xylosylated N-glycans (insect cells), respectively. Data from preliminary Western blots indicate the potential of insect cells to xylosylate N-glycans of endogenous proteins, as soon as a recombinant ß1,2-xylosyltransferase from *Arabidopsis thaliana* is expressed. Having modified glycoproteins on the cell surface, FACS will be used for sorting those clones expressing the ß1,2-xylosyltransferase from gastropods. In parallel, the ß1,2-xylosyltransferase is isolated from a microsomal fraction of snail tissue through several chromatographic steps. This project is supported by the FWF project number P22118-B20.

312: A vertebrate-specific Y subfamily of UDP-N-acetyl-α-D-galactosamine: polypeptide N-acetyl-galactosaminyltransferases

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O-Linked glycosylation of mucin is initiated by the UDP-N-acetyl-α-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase (ppGalNAc-T) family, which transfer N-acetylgalactosamine with an α-linkage to the serine or threonine residue in protein. ppGalNAc-Ts are type II membrane proteins and to date, 20 distinct ppGalNAc-T family members have been identified in human. Recently, a new subfamily of ppGalNAc-Ts, designated as Y subfamily, has been identified and characterized. The Y subfamily consists of four members, ppGalNAc-T8, -T9, -T17, and -T18 in which the conservative $YDX_5WGGENXE$ sequence in Gal/GalNAc-T motif of ppGalNAc-Ts mutated to $LDX_5YGGENXE$. Phylogenetic analysis revealed that Y subfamily members only exist in vertebrates. All four Y

subfamily members lack the classical in vitro GalNAc-transferase activity which might result from the UDP-GalNAc binding pocket mutations by structural homology modeling analysis. Finally, the new defining member of the Y subfamily, ppGalNAc-T18, was found to localize in the ER rather than Golgi apparatus in lung carcinoma cells. These results suggest that the Y subfamily ppGalNAc-Ts particularly distinguish themselves from the W subfamily members and they may play distinct functions in vertebrates.

313: Insights into evolutionary history of animal β-galactoside α2,6-sialyltransferases that explain ubiquitous expression of st6gal1 gene in Amniotes

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The animal sialyltransferases (ST) superfamily comprises 4 ST families (ST3Gal, ST6Gal, ST6GalNAc and ST8Sia) and 20 ST subfamilies in higher vertebrates that catalyze the transfer of sialic acid residues. We have recently identified the 4 families in invertebrate genome suggesting ancient occurrence and divergent evolution of STs [1]. ST6Gal-I and ST6Gal-II are the two unique members of the ST6Gal family described in higher vertebrates. Focussing on the st6gal gene family, we confirmed the occurrence of a unique ancestral st6gal gene in the sponge *O. carmela*, early in the Metazoan lineage. Two subsets of st6gal genes (st6gal1 and st6gal2) arose from the second round of whole genome duplication, about 450 MYA. Using a phylogenomic approach, we present further evidence of an accelerated evolution of the st6gal1 genes both in their genomic regulatory sequences and in their coding sequence in reptiles, birds and mammals known as amniotes. Our synteny studies and gene organization analysis pointed out a disruption in the conservation of the synteny of st6gal1 gene in fish genome, suggesting a translocation event of this gene early after teleosts divergence. Finally, we found that st6gal1 gene expression in vertebrate tissues is not phylogenetically conserved, correlating with modifications observed in the 5'end of the gene, whereas st6gal2 genes conserve an ancestral profile of expression throughout vertebrate evolution. Altogether, our study suggests that the vertebrate ST6Gal-I progressively acquired functions in the immune system from a probable ancestral role in embryonic and adult central nervous system that has been maintained by ST6Gal-II [2].

References: [1] Harduin-Lepers A et al. 2005 Glycobiology 15, 805; [2] Petit et al. 2010 J. Biol. Chem. 285, 38399.

314: N-glycosyltransferase Complexes Rather than Enzyme Monomers Are Responsible for the Processing and Terminal Maturation of N-Glycans in the Golgi of Live Cells

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Glycans attached to cellular proteins and lipids come in many varieties and much of this diversity between molecules, cells and tissues is generated by Golgi-resident glycosidases and glycosyltransferases that operate in the different Golgi glycosylation pathways. Here we investigate the supramolecular organization of the Golgi N-glycosyltransferases in live cells by utilizing the BiFC approach. We found that all the main N-glycosylation enzymes tested (GnTI, GnTII, GalT, SiaT) form not only Golgi-localized homomeric enzyme complexes but also heteromeric complexes, one between the medial-Golgi enzymes and the other between the trans-Golgi enzymes. In vitro and in vivo enzyme activity measurements showed that the enzyme complexes were more active than the homomeric complexes.

Collectively, these data suggest that Golgi N-glycosyltransferases assemble into functionally relevant heteromeric, dimeric, or even oligomeric enzyme complexes, and that these complexes are responsible for the processing and terminal maturation of N-glycans in live cells.

315: Alg14 organizes the formation of a multi-glycosyltransferase complex required for initiation of lipid-linked oligosaccharide biosynthesis

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Protein N-glycosylation begins with the assembly of a lipid-linked oligosaccharide (LLO) on the endoplasmic reticulum (ER) membrane. The first two steps of LLO biosynthesis are catalyzed by a functional multienzyme complex comprised of the Alg7 GlcNAc phosphotransferase and the heterodimeric Alg13/Alg14 UDP-GlcNAc transferase on the cytosolic face of the ER. In the Alg13/14 glycosyltransferase, Alg14 recruits cytosolic Alg13 to the ER membrane through interaction between their C-termini. Bioinformatic analysis revealed that eukaryotic Alg14 contains an evolved N-terminal region that is missing in bacterial orthologues. Here, we show that this N-terminal region of *Saccharomyces cerevisiae* Alg14 localize its GFP-fusion to the ER membrane. Deletion of this region causes growth defect at a high temperature (38.5) that can be partially complemented by overexpression of Alg7. Coimmunoprecipitation demonstrated that the N-terminal region of Alg14 is required for direct interaction with

Alg7. Our data also show that Alg14 lacking the N-terminal region remains on the ER membrane through a nonperipheral association. Mutational studies guided by the 3D structure of Alg14 indentified a hydrophobic interface working as another membrane-binding site. We propose a model in which the N- and C-termini of Alg14 coordinate recruitment of catalytic Alg7 and Alg13 to the ER membrane for initiating the LLO biosynthesis.

316: *Arabidopsis thaliana* ALG10 is required for efficient N-glycosylation and leaf development

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Assembly of the dolichol-linked oligosaccharide ($Glc_3Man_9GlcNAc_2$) is highly conserved among eukaryotes. In contrast to yeast and mammals little is known about the biosynthesis of dolichol-linked oligosaccharides and the transfer to asparagine residues of nascent polypeptides in plants. To understand the biological function of these processes for plants we characterized the *Arabidopsis thaliana* homologue of yeast ALG10, the α1,2-glucosyltransferase that transfers the terminal glucose residue to the lipid-linked precursor. Expression of an Arabidopsis ALG10-GFP fusion protein in *N. benthamiana* leaf epidermal cells revealed a reticular distribution pattern resembling endoplasmic reticulum localization. The analysis of lipid-linked oligosaccharides showed that Arabidopsis ALG10 can complement the yeast alg10 mutant strain. A homozygous Arabidopsis T-DNA insertion mutant (alg10-1) accumulated mainly lipid-linked $Glc_2Man_9GlcNAc_2$ and displayed a severe underglycosylation defect. Phenotypic analysis of alg10-1 showed that mutant plants have altered leaf morphology when grown on soil. Moreover, the inactivation of ALG10 in Arabidopsis resulted in the activation of the unfolded protein response and increased salt sensitivity. In summary, these data show that Arabidopsis ALG10 is an ER-resident α1,2-glucosyltransferase that is required for lipid-linked oligosaccharide biosynthesis and subsequently for normal leaf development and abiotic stress response.

317: O-glycosylation pathways in human corneal and conjunctival cells

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Cells on the surface of the eye produce a number of mucins with protective functions. Diseases associated with alterations in mucin glycosylation include dry eye, a common ocular pathology characterized by inflammation and a higher concentration of the inflammatory cytokine tumor necrosis factor-alpha (TNFα). In this work, we have examined the pathways of mucin type O-glycosylation in mucin-producing human corneal and conjunctival epithelial cells, and evaluated the effect of TNFα on glycosyltransferase activities. The results show that both corneal and conjunctival ocular surface epithelial cells have active enzymes that synthesize O-glycans with the common core structure 1 (Galβ1-3GalNAc-) and core 2 (GlcNAcβ1-6(Galβ1-3)GalNAc-). Treatment of cells with TNFalpha altered the activity of a number of glycosyltransferases in a dose-dependent and cell type-specific manner, suggesting that mucin O-glycosylation changes in a dynamic fashion during inflammatory stimuli. This work was funded by an NIH grant (PA) and the Canadian Cystic Fibrosis Foundation (IB).

318: Involvement of Rot1 in protein N- and O-glycosylation in *Saccharomyces cerevisiae*

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Rot1 is an essential yeast protein and is related to such processes as cell cycle, cell wall biosynthesis, actin cytoskeleton dynamics and protein folding. Rot1 is an N-glycosylated protein, which is anchored to the nuclear envelope-endoplasmic reticulum (ER) membrane by a transmembrane domain at its C-terminal end and is translocated to the ER by a post-translational import mechanism. In previous experiments we could demonstrate that overexpression of ROT1 suppresses the temperature-sensitive phenotype of sec59-1 mutant, impaired in dolichol kinase (Sec59). Here we show that Rot1 interacts in a synthetic manner with Ost3, one of the nine subunits of the oligosaccharyltransferase complex, the key enzyme of N-glycosylation. Deletion of OST3 in the rot1-1 mutant causes a temperature-sensitive phenotype as well as sensitivity towards Calcofluor White, caffeine, Congo Red and hygromycin B, whereas deletion of OST6, a functional homologue of OST3, has no effect. Oligosaccharyltransferase activity in vitro was found to be decreased to 40 % compared to wild-type membranes, and model glycoproteins for N-glycosylation, like carboxypeptidase CPY, Gas1 or DPAP B, displayed an underglycosylation pattern. Furthermore an aberrant cell wall composition was detected. Surprisingly, for the first time also a role of Rot1 in O-mannosylation, e.g. of chitinase, could be demonstrated. Altogether, our results indicate that Rot1 interacts with the Ost3 subunit of oligosaccharyltransferase and plays a role in N- glycosylation and O-mannosylation of at least some of the proteins.

319: The effect of MSMEG_6402 gene disruption on the cell wall and morphology of *Mycobacterium smegmatis*

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Arabinogalactan (AG) of mycobacterial cell wall consists of arabinan region, galactan region and disaccharide linker. The arabinan is composed of D-arabinofuranose residues and decaprenyphosphoryl-D-arabinose (DPA) is the donor of the D-arabinofuranose residues. DPA is formed from phosphoribose diphosphate (PRPP) in a four-step process catalyzed by transferase, phosphatase and epimerase, respectively. *M. tuberculosis* Rv3806c has been identified as PRPP: decaprenyl-phosphate 5-phosphoribosyltransferase and heteromeric Rv3790/ Rv3791 has epimerase activity. Rv3807c is putative phospholipid phosphatase. However, there is no direct biochemical evidence since expression of Rv3807c has been unsuccessful.

M. smegmatis MSMEG_6402 is ortholog of Rv3807c. To investigate the function of MSMEG_6402 on AG biosynthesis we constructed a conditional MSMEG_6402 gene knock out (ΔMSMEG_6402) strain through homologous recombination technique. The morphological change and chemical composition were examined in the ΔMSMEG_6402 strain, respectively. The ΔMSMEG_6402 strain grew at non-permissive temperature slower than that at permissive temperature, indicating that MSMEG_6402 is non-essential for growth of *M. smegmatis*. The change of cell shape and obvious bulging on the cell surface of ΔMSMEG_6402 strain were observed by scanning electron microscopy. The results by transmission electron microscopy showed curled and deformed cell wall of ΔMSMEG_6402 strain. Analysis of sugar composition by HPLC indicated that the ratio of Ara to Gal in the cell wall of ΔMSMEG_6402 strain was changed to 1.2:1, whereas, the ratio of Ara to Gal in the cell wall of wild type *M. smegmatis* is 2:1. It demonstrates that the lacking MSMEG_6402 interferes the biosynthesis of arabinan. Analyzing 5' P-DPR and DPR from both ΔMSMEG_6402 strain and wild type M. smegmatis is under way in our lab.

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320: Elucidation of the N-glycosylation pathway in the thermoacidophilic archaeon *Sulfolobus acidocaldarius*

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Historically it was long been believed that glycosylation is a unique phenomena restricted to Eukarya. Today N-glycosylation is thought to be conserved across all three major domains of life. During the last years substantial progress in describing N-glycosylation pathways in three different euryarchaeota, one of the major archaeal kingdoms, has been made. However, the N-glycosylation process in the more ancient kingdom, the crenarchaeota is still uncovered. Here we will report the first results elucidating the N-glycosylation pathway in the thermoacidophilic archaeon *Sulfolobus acidocaldariu*s. The S-layer proteins SlaA, used as a reporter glycoprotein, possesses a remarkably high glycosylation density. In only the C-terminal part of the protein at least 9 of 11 glycosylation motifs were found to be glycosylated. Each of the glycosylation sites observed was shown to be modified with a heterogeneous family of glycans, with the largest composed of a tribranched hexasaccharide (Glc-QuiS)-(Man)-(Man)-$GlcNAc_2$. Sulfoquinovose (QuiS) is so far only found as the head group of the nonphosphorous lipid sulfoquinovosyldiacylglycerol located exclusively in the photosynthetic membrane of all plants and of most photosynthetic bacteria, further *S. acidocaldarius* is the only archaeal species whose N-glycans are known to be linked via the chitobiose core disaccharide that characterises the N-linked glycans of Eukarya. Here we will also report of the first identified enzyme (UDP-sulfoquinovose-synthase) participation in the N-glycosylation pathway, by activating the sulfoquinovose as a precursor for the assembly of the N-glycan. A markerless deletion mutant of this gene, revealed a reduced N-linked glycan on the S-layer composed of only the unbranched trisaccharide Man_1-$GlcNAc_2$.

321: Overexpression of *M. tuberculosis* GlcNAc-1-phosphate transferase in *E. coli* ER2566

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The cell wall core of mycobacteria includes mycolic acid, arabinogalactan, and peptidoglycan. The mycolic acid-arabinogalactan is connected to the peptidoglycan by a disaccharide linker, D-N-acetylglucosamine-L-rhamnose. Therefore, the disaccharide linker is fundamental to the structural integrity of the cell wall. The first step of the

disaccharide linker is formation of decaprenyl phosphate-GlcNAc, which is catalyzed by GlcNAc-1-phosphate transferase. *E. coli* wecA gene specifies the UDP-GlcNAc: undecaprenyl phosphate GlcNAc-1-phosphate transferase (WecA) that catalyzes the first step in the biosynthesis of lipopolysaccharide (LPS) O-antigen. *E. coli* MV501, a wecA defective strain, can not initiate O-antigen biosynthesis. In our previous study, we cloned *M. tuberculosis* Rv1302 and transformed MV501 with the plasmid carrying *M. tuberculosis* Rv1302. The result showed that LPS synthesis in MV501 was restored upon complementation with *M. tuberculosis* Rv1302. Therefore, *M. tuberculosis* Rv1302 is wecA gene to encode GlcNAc-1-phosphate transferase (WecA). *M. tuberculosis* WecA protein was predicted as membrane protein having 11 trans-membrane domains. In this study, we constructed an expression plasmid pET16b-Mtb wecA and transformed pET16b-Mtb wecA to *E. coli* ER2566. ER2566/pET16b-Mtb wecA was induced with IPTG at room temperature and the membrane fraction containing *M. tuberculosis* WecA protein was prepared by ultracentrifugation. *M. tuberculosis* WecA protein was solublized by detergent DDM and purified by Ni-NTA. The purified *M. tuberculosis* WecA protein was confirmed by anti-His-tag antibody. The assay of GlcNAc-1-phosphate transferase was established and the purified *M. tuberculosis* WecA protein showed activity of GlcNAc-1-phosphate transferase. The kinetic parameters of GlcNAc-1-phosphate transferase will be determined in further study. This work was supported by the National Basic Research Program of China (2006CB504400) and Key Project of Major Infectious Diseases (2008ZX10003-006)

322: Chemoselective Synthesis of Artificial Glycoproteins for Multivalent Lectin Binding Studies

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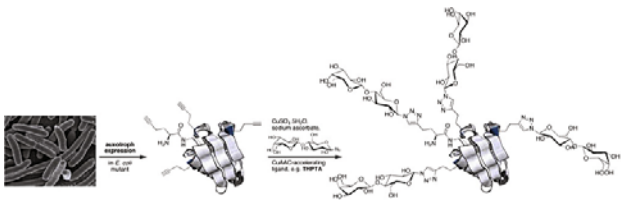
In the context of the growing biological and pharmaceutical interest in carbohydrate-binding proteins, namely lectins, and their mode of interaction, synthetic strategies for the functionalization of multivalent target scaffolds are highly desirable.[1],[2] With this background, we aim to use proteins as synthetic scaffolds for the presentation of different carbohydrate moieties on its surface to probe their efficiency in multivalent lectin-binding studies. Although purely synthetic strategies such as the combination of solid phase peptide synthesis (SPPS) and native chemical ligation (NCL) as well as intein-splicing are available,[3] those methods have the drawback of being sequence-specific and time consuming. A different method to obtain proteins would be the biosynthesis. Incorporation of non-canonical amino acids (NCAA) into a protein sequence represent a suitable basis for further posttranslational and bio-orthogonal modifications, e.g. copper-catalyzed azide-alkyne cycloaddition or Staudinger-phosphite reaction.[4],[5] In order to equip model proteins with alkyne-containing NCAAs, genetic code expansion or genetic code engineering methods can be used. Briefly, genetic code engineering means that a methionine (Met) auxotrophic *E. coli* strain was supplemented with the Met analog homopropargyl glycine (Hpg) just before the expression of the target protein was induced. After purification, a highly pure and homogeneous protein was analytically characterized, conferring almost quantitative Met -> Hpg substitution level in a residue-specific manner. This is in contrast to the genetic code expansion, where only one amino acid will be replaced.[5]

Herein we present our resent results on the elucidation of the multivalent binding mode of lectins with the use of artificial glyco-proteins.



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323: Functional and structural characterization of a flavonoid glucoside 1,6-glucosyltransferase from *Catharanthus roseus*

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In higher plants secondary metabolites are often converted to their glycoconjugates by UDP-sugar-dependent glycosyltransferases. Since glycosyl conjugation of low molecular weight compounds enhances water solubility, improves chemical stability, and alters physiological activity, glycosylation using recombinant glycosyltransferases has attracted considerable research interest. Much efforts has recently gone into cloning and characterization of a substantial set of plant

glycosyltransferases that transfers sugar molecules to the OH and/or COOH group of aglycones. In contrast, few studies has centered on sugar-sugar glycosyltransferases which catalyse sugar conjugation to sugar moiety of plant glycosides in spite of their important role in increasing structural diversity of small molecule glycosides in higher plants. We isolated a cDNA clone encoding a sugar-sugar glucosyltransferase (UGT94E3) catalyzing 1,6-glucosylation of flavonol- and flavone glucosides for the first time from *Catharanthus roseus*. UGT94E3 exhibited a unique glucosyl chain elongation activity forming not only gentiobioside but also gentiotrioside and gentiotetroside in a sequential manner. Expression analysis suggested that UGT94E3 may be involved in biosynthesis of defence-related flavonoid glycosides in the leaf tissues of *C. roseus*. In addition, we investigated the functional properties of UGT94E3 using homology modeling and site-directed mutagenesis and identified amino acids positioned in the acceptor binding pocket as crucial for providing enough space to accommodate flavonoid glucosides instead of flavonoid aglycones. These results provide basic information for understanding and engineering the catalytic functions of sugar-sugar glycosyltransferases involved in biosynthesis of plant glycosides.

324: Effect of grazing time and feeding in the concentration of sialic acid and hexose in bovine milk

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Sialic acid, which is located at the terminal end of glycoconjugates, is believed to have important biological functions. Its concentration in bovine milk varies depending on lactation stage and season. However, it remains unclear whether or not dietary factors, especially fresh forage, affect the total sialic acid concentration in the milk. The purpose of the present study was to investigate the effect of grazing time and feeding on the concentrations of total sialic acid and hexose in bovine milk. In the first study, eight healthy dairy cows were used in the grazing experiment for 1 week. In the other experiment, six healthy dairy cows were used in a crossover design (3 cows fed fresh forage and 3 cows fed grass silage) for 2 weeks. Individual milk samples were collected at after 1 week in the grazing experiment, and at 0, 1, 3, 5, 8, 11, and 14 d of the crossover experimental period. There was no difference in the hexose concentration in bovine milk. On the other hand, the level of toatal sialic acid in each grazing time for 1 week were significantly higher than non-grazing cow's milk. Moreover, the total sialic acid concentration in the milk of each grazing cow significantly increased at 11 and 14 d of the experimental period compared with that at 0 d. These results indicate that grazing management could have increased the concentration of sialoglycoconjugates in milk. This suggests that grazing may increase the biological function of milk, because it is thought that sialic acid is significant in some different manner.

325: Identification of key functional residues in the active site of human β1,4-galactosyltransferase 7: a major enzyme in glycosaminoglycan synthesis pathway

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Glycosaminoglycans (GAG) are polysaccharide chains attached to the core protein of proteoglycans, playing vital biological functions. β1,4-galactosyltransferase 7 (β4GalT7) is a key glycosyltransferase involved in the initiation of GAG chain synthesis. This enzyme is also able to use exogenous xyloside derivatives as substrates with interesting biomedical applications in the treatment of pathologies associated with alterations of GAG synthesis. In this context, we performed a structural and functional study of the human recombinant β4GalT7 and built a molecular model of this protein, based on the 3D structure of Drosophila β4GalT7. Phylogenetic analyses of the β4GalT7 family and multiple sequence alignment showed the presence of highly conserved motifs, notably the ^{163}DVD^{156} and ^{221}FWGWGREDDE^{230} sequences, potentially involved in the active site organization. In an original way, we combined in vitro and ex vivo approaches to assess the role of each amino acid residue of these two conserved motifs. The study of the consequences of site-directed mutations on kinetic and functional properties of the human β4GalT7 allowed us to identify key active site amino acids. Indeed, our work demonstrated that Trp224 is involved in donor and acceptor substrate interactions and that Asp165 forms coordination bonds with Mn^{2+} cations. We also suggested Asp228 as the catalytic base of the human β4GalT7. Interestingly, the W224H mutation enhanced enzyme affinity towards the donor substrate UDP-Gal, and also modulated the specificity of this enzyme towards the acceptor substrate. Furthermore, our ex vivo study showed that the W224H mutant is able to restore GAG chain substitution on the core protein of the human recombinant decorin, but not on exogenous

xyloside acceptors. Altogether, this work establishes an important headway in the understanding of GAG biosynthesis pathway and opens new perspectives for the development of innovative therapeutic strategies based on the use of exogenous xyloside derivatives in the treatment of the pathologies owed to GAG biosynthesis aberrations. J.Biol.Chem. 2010. 285(48):37342-58

326: Involvement of Golgi localised nucleotide sugar transporters in *Aspergillus fumigatus* galactomannan biosynthesis

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The human pathogen *Aspergillus fumigatus* causes the life-threatening disease invasive aspergillosis affecting immunocompromised patients. Current antifungals primarily target plasma membrane components or cell wall biogenesis. Treatment options are however limited and emerging resistance emphasises an urgent need for the definition of new drug targets. A major component of the *A. fumigatus* cell wall is galactomannan, a unique polysaccharide composed of a linear mannan with galactofuranose (Gal*f*) side-chains. The biosynthesis and biological role of this polysaccharide are currently poorly defined. We have, however, previously demonstrated that the absence of Gal*f* leads to a growth defect, a thinner cell wall and attenuation of the virulence of *A. fumigatus*. Recently we have identified a Golgi localised UDP-Gal*f* transporter and demonstrated by targeted gene deletion that it controls galactofuranosylation of the cell wall. We therefore propose a model in which galactomannan is first assembled on a lipid anchor along the ER/Golgi pathway and then transferred to the cell wall by transglycosylation. This model is supported by reports of a GPI-linked galactomannan in *A. fumigatus*, but contrasts with the biosynthesis of the fungal cell wall components chitin and glucan that occurs directly at the plasma membrane. According to this hypothesis, assembly of galactomannan would thus also depend on import of GDP-mannose into secretory vesicles. First results about the identification and characterisation of the *A. fumigatus* GDP-Man transporter will be presented. Moreover, the involvement of this transporter in glycolipid and galactomannan biosynthesis was investigated in a deletion mutant severely affected in viability.

327: Analysis of in vitro activities of human ER α-1,2 mannosidase I on denatured glycoproteins

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Endoplasmic reticulum (ER) α-1,2 mannosidase I is an enzyme which removes α-1,2 linked mannoses from asparagine-linked oligosaccharides on glycoproteins in ER. The mannosidase I preferentially removes one α-1,2 linked mannose from B-chain of $Man_9GlcNAc_2$, producing $Man_8GlcNAc_2$-B. When glycoproteins failed to be properly folded, increased removal of α-1,2 linked mannoses on asparagine-linked oligosaccharides on those glycoproteins was induced to dispose those glycoproteins from ER to the cytosol and degrade them by ER-assisted degradation pathway. However, it is still inconclusive whether accelerated removal of α-1,2 linked mannoses on those glycoproteins was catalyzed by the α-1,2 mannosidase I, proteins similar to mannosidase I [e.g. EDEM (ER degradation-enhancing α-1,2-mannosidase-like protein)], or both of them. Therefore, to approach this issue, we have investigated its activities in vitro using various oligosaccharides and glycoproteins as substrates.

A portion of catalytic domain of human mannosidase I with 6xhistidine tag was expressed in *Escherichia coli*. The polypeptide was purified by nickel-bound resin and used as an enzyme to analyze its catalytic properties on oligosaccharides and glycoproteins. First, when the enzyme was incubated with 100 μM pyridylamine-conjugated $Man_9GlcNAc_2$ ($Man_9GlcNAc_2$-PA) at 37 °C, $Man_6GlcNAc_2$-PA and $Man_5GlcNAc_2$-PA were detected after a one-hour reaction. This demonstrated that the enzyme could remove four or five α-1,2 linked mannoses in the short period. Second, we have exposed soy bean agglutinins and bovine thyroglobulins to a denaturing condition, e.g. 8 M urea, and used those glycoproteins as substrates. Sugar moieties were released from the reactant by PNGase F and their structures and amounts were elucidated by HPLC analysis. Intriguingly, the enzyme was shown to remove mannoses from soy bean agglutinins and bovine thyroglobulins to larger extents when they were exposed to a denaturant than those glycoproteins without any treatments. Therefore, our results suggested that human ER α-1,2 mannosidase I could recognize tertiary structures of glycoproteins and removed more α-1,2 linked mannoses from misfolded glycoproteins in ER.

328: Characterization of PNGase-independent pathways for free-oligosaccharide formation and processing in mammalian cells

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Free oligosaccharides (fOSs) are known to be generated in the cytosol from misfolded glycoproteins by action of peptide:N-glycanase (PNGase) in various organisms [1]. In mammalian cells, the fOSs are also formed from lipid-linked oligosaccharides (LLOs) by unidentified mechanisms on the luminal and/or cytosolic surfaces of

the endoplasmic reticulum (ER) membrane [1]. It has been reported that LLOs are cleaved at two distinct sites: the pyrophosphate bond and the N-acetylglucosamine-phosphate bond [2,3,4]. Genes encoding enzymes involved in these processes have not been identified. In this presentation, we will report our most recent progress on characterization of enzymes involved in the PNGase-independent formation and processing of fOSs. References: [1] Suzuki, T. Sem. Cell Dev. Biol. 17, 762-769 (2007). [2] Gao, N., et al. J. Biol. Chem. 280:17901-17909 (2005). [3] Peric, D., et al. PLoS One 5(7):e11675 (2010). [4] Vleugels, W., et al. Biochimie 93, in press (2011).

329: Post-Translational Regulation of Glycolipid-Glycosyltransferase Expressions in Apoptotic Breast and Colon Carcinoma Cells

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Cell surface glycoconjugates (glycolipids and glycoproteins) are altered during metastasis of breast as well as colon cancer cells. However, the relationship between their structural alterations and the degree of metastasis is not well understood. Previously, we have characterized expression of all six different glycolipid-glycosyltransferases (GalT-2, GalT-4, GalT-5, SAT-2, SAT-3, and FucT-3) in both colon (Colo-205) and three different breast carcinoma lines. They were cloned from highly metastatic cancer cells. During our investigation with different apoptotic agents: L-PPMP (1 to 16 micromolar) and D-PDMP (1 to 16 micromolar) (both are inhibitors of glycolipid biosynthesis) and cis-platin (an inhibitor of DNA biosynthesis), and betulinic acid (a herbal drug used for cancer treatment in China; 10 to 80 micromolar) we quantitated a dose-dependent reduction of GLT-activities in these cell lines after 6 to 72 hours of drug treatment. These observations suggest that, during the apoptotic process with the activation of specific Caspases (-3, -8, and -9) perhaps some nonspecific proteases are also stimulated for inactivation of GLTs. Using a membrane- specific fluorescent dye (AKS-O), drastic damages in both mitochondrial and Golgi membranes were observed during these apoptotic processes.

330: Extensive incorporation and recycling of environmental glycoprotein-conjugated sialic acids by tumor cells: proposed mechanism based on new findings obtained by chemical analysis

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Sialic acids (Sias) are unique sugar residues that occupy terminal positions of oligosaccharide chains of cell surface glycoconjugates. Qualitative and quantitative changes of them may change cellular interaction and recognition phenomena in neoplasm, and may play a role in malignancy of tumor cells. Neu5Gc (N-glycolylneuraminic acid) is a non-human sialic acid. Neu5Gc-containing glycans are known as cancer-specific HD-antigens. During our studies analyzing tumor-associated changes in sialic acids, we observed that tumor cells extensively incorporated environmental Sias into soluble sialoglycoproteins that were synthesized and secreted by the cells. As the result, in some human tumor cells, cultured in the presence of fetal calf serum (FCS), the proportion of glycoprotein-bound Neu5Gc was > 20% of total Sia. The secreted sialoglycoproteins were fixed on culture dishes, and appeared to re-enter into the cell, and re-processed. High Neu5Gc/Neu5Ac ratios were recorded in cells in which de novo sialic acid synthesis was poor. Free Neu5Gc that occurs in minute amount in FCS may not be the major source of Neu5Gc incorporated in tumor glycoproteins. Rather, environmental Neu5Gc-containing glycoprotein(s) accumulated nearby the growing cells, initially sialoglycoprotein(s) in FCS, and Neu5Gc-enriched sialoglycoproteins secreted by the cells during cell growth may be major sources of Neu5Gc. Recycling of Sias, and extensive enrichment of Neu5Gc may involve new biosynthetic and degradation mechanism of sialoglycoproteins by tumor cells. Elucidation of the mechanism, and characterization of Neu5Gc enriched-glycoproteins are our ongoing projects. [Sadako Inoue et al. (2010) Glycobiology 20, 752-762]

331: Newly-identified Receptors for Pancreatic α-Amylase Control Blood Glucose Concentration via Carbohydrate-specific Interactions

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We previously reported that porcine pancreatic a-amylase (PPA) binds to N-linked sugar chains of glycoproteins [1] and that recombinant human pancreatic α-amylase shares the N-glycan-binding activity. In this study, we identified the endogenous ligand glycoproteins for PPA in the duodenum and found functional changes in the ligands due to carbohydrate-specific interaction with PPA. PPA found at the surface of enterocytes of the brush-border membrane (BBM) in the duodenum by immunostaining was completely released by treatment of the duodenum with methyl α-mannoside (Me α-Man), indicating that PPA binds specifically to carbohydrates in BBM. Possible glycoligands for PPA were screened by combining affinity chromatography using a PPA-coupled Sepharose column,

PAGE, and LC/MS. The PPA ligands in BBM were identified as N-glycoproteins that are involved in the assimilation of glucose, including sucrase-isomaltase (SI) and Na^+/Glc cotransporter 1 (SGLT1). ELISA using an antibody for each protein showed that they interacted with PPA mannose-specifically. The amylolytic activity of PPA was enhanced in the presence of Me α-Man, N-glycosylated glycoproteins, or BBM. The maltose-hydrolyzing activity of SI was elevated in the presence of PPA. On the other hand, glucose transport by SGLT1 into reconstituted BBM vesicles was markedly inhibited by PPA, but recovered by the addition of N-glycan-specific plant lectins, indicating that the effect was caused by carbohydrate-specific binding of PPA to SGLT1. Thus, (1) PPA is located in BBM through binding to high-mannose type or complex type sugar chain of glycoproteins in BBM, (2) the binding remarkably activates starch-hydrolysis of PPA while suppresses Glc incorporation into enterocytes, and (3) PPA differentially regulates each activity of BBM ligands. The results strongly demonstrate that N-glycans on BBM glycoproteins work not only as the target signal for PPA localization in porcine duodenum, but that the PPA receptors control the blood glucose concentration by a novel carbohydrate-specific mechanism. Reference: [1] Matsushita, H., et al. J. Biol. Chem., 277, 4680-4686 (2002)

Poster Session II (Thursday)

Structural & Chemical Glycobiology and Glycomics

332: O-glycan profiling for rat gastric mucins by MALDI-TOF/MS and HPLC

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The mucin-type O-glycan seems to have important role in protecting gastric mucosa by mucins against various aggressive factors. Histochemical studies show the different expression patterns of mucin glycans among the gastric regions, for example, the corpus and antral mucosa. Here, we examined the O-glycan profile for the rat gastric mucins. Rat gastric mucins (RGM) were prepared from corpus and antral mucosa of Wistar rat stomach. Mucin-type O-glycans were prepared and analyzed using two methods. Firstly, the reduced-form O-glycans were obtained from RGM by alkaline-borohydride treatment and after permethylation analyzed by MALDI-TOF/MS and MS/MS. Secondly, the O-glycans bearing reducing terminal GalNAc as an intact form were obtained from RGM by treatment with anhydrous hydrazine and, after derivatized with anthranilic acid, they were separated by HPLC using an amide column (1) and fractionated. Each O-glycan was characterized by MALDI-TOF/MS and MS/MS. More than 20 major O-glycans were obtained from both corpus and antral mucins by alkaline-borohydride treatment. Corpus and antral mucins showed similar glycosylation patterns, but several glycans were characteristic in each mucin. Both mucins contained several O-glycan having peripheral HexNAc, some of which seemed to be the glandular mucin-specific glycans bearing peripheral α4-linked GlcNAc (2). The O-glycan obtained by hydrazine treatment contained many degraded products, which were produced by the "peeling" reaction, as described previously (1). However, their structures bring the important information that some O-glycans of the rat gastric mucin possess a relatively long core 1-side branch. (1) Goso et al. (2009) J. Biochem., 145, 739 - 749. (2) Ishihara et al. (1996) Biochem. J., 318, 409 - 416.

333: Top-down and Bottom-up Approaches to Understanding Glycan Processing in the ER

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Glycoprotein oligosaccharides in the endoplasmic reticulum (ER) function as tags for protein quality control. Since misfolded protein accumulation caused by failure of the quality control correlates with various folding diseases, we speculate that glycan-profiles in the ER might be useful index to give individual cellular state. To understand glycan processing in the ER, we have been working on substrate specificity analysis of several glycan-processing enzymes using synthetic oligosaccharide substrates [1]. Although these synthetic bottom-up approaches gave clear results in most cases, the in vitro experiments gave partially distinct results from that of in vivo experiments. Thus we expanded our approach to a pilot-study of connecting in vitro and in vivo as a second-generation bottom-up approach. Namely, we investigated glycan-processing analysis under pseudo intracellular environments attending to molecular crowding [2] and micro-space effects. As a result, we found partially different glycan-processing properties compared with that obtained by the first generation bottom-up approaches. On the other hand, we also attend to meaning of the ER glycan-profiles, the balance of glycoforms, as a top-down approach to understand the glycan function. The direct glycan-analyses of ER-extracts are often hampered by retro-translocated glycoforms from Golgi and cytosol. To overcome this probrem, we reconstructed the ER glycan-processing using ER-extracts as enzymatic source and synthetic glyco-probe as a substrate. The resulting glycan-profiles from model-rats of diabetes, the example of folding disease, clearly showed different aspects compared to those obtained from healthy rats as negative control. The bottom-up approach gives molecular-basis for glycan function, and the top-down approach gives meaning of

glycan-profiles. Thus, the synthetic glyco-probe connecting two-way approaches will be effective to molecular levels understanding of functions of glycan-processing in the ER. References: [1] Takeda, Y. et al. Y. Curr. Opin. Chem. Biol. 2009, 13, 582-591; [2] Totani, K. et al. Y. J. Am. Chem. Soc. 2008, 130, 2101-2107.

334: The ER glycan processing profile correlates with type II diabetes

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Correct folding of glycoproteins are necessary for their function. This will be facilitated by the endoplasmic reticulum protein quality control system (ERQC) [1]. During ERQC, each glycoform possessing on the protein surface has been known to function as a tag for its folding, sorting, secretion and degradation. Namely, ERQC is correlated with ER glycan profiles. On the other hand, breaking of ERQC by multiple disturbances causes accumulation of misfolded protein called ER stress. These circumstances would cause folding disease such as Alzheimer disease and diabetes[2].

In this work, we investigated the correlation between type II diabetes and glycoprotein glycan profiles in the ER in order to understand the folding diseases by specific balance of the glycoforms. The conventional glycan analysis, however, could not extract the feature of diabetes. Thereby, we turn out reconstruction of the glycan profiles using synthetic glycans and the ER fraction from rat liver. We investigated the glycan profiles on calnexin/calreticulin (CNX/CRT) cycle containing protein folding, and the mannose trimmings that correlate with protein secretion and degradation. However, ER essential folding sensor enzyme UGGT only accepts Molten globule (MG) glycoproteins. Poor reproducibility of conformation of the MG glycoproteins makes it difficult to carry out quantitative analysis of UGGT. To overcome this, we have reported glycan-BODIPY conjugates as MG glycoprotein-mimetics having homogeneous oligosaccharide. As a result, different glycan profiles were obtained from Goto-Kakizaki rat (GK) as a model of diabetes and Wistar rat (WT) as a negative control. Moreover we investigated activity of each glycan processing enzymes. The differences of these glycan profiles are expected to be useful as novel types of disease marker, and also may give a speculation to understand the pathogenic mechanism of diabetes. References: [1] T. Cali, O. Vanoni, M. Molinari, Prog. Mol. Biol. Transl. Sci., 83, 135 (2008); [2] H.Yoshiba, FEBS J., 274, 630 (2007)

335: Structural analysis of the Duffy atypical chemokine receptor N-linked oligosaccharide chains

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Duffy antigen/receptor for chemokines (DARC) is a glycosylated seven-transmembrane protein acting as a blood group antigen, a chemokine binding protein and a receptor for *Plasmodium vivax* malaria parasite. It is present on erythrocytes and vascular endothelial cells. DARC binds several proinflammatory CC and CXC chemokines but it has no ability for signal transduction, therefore it is described as an atypical chemokine receptor (ACR).

N-terminal extracellular domain of the Duffy glycoprotein carries Fy^a/Fy^b blood group antigens, Fy6 linear epitope recognized by monoclonal antibodies and three potential *N*-glycosylation sites at Asn16, Asn27 and Asn33. Detailed structures of the oligosaccharide chains of DARC or other chemokine receptors have not been determined so far. Previously we have reported that recombinant Duffy protein expressed in K562 cells has three *N*-linked oligosaccharide chains which are mostly of complex type, rich in *N*-acetyllactosamine units terminating with α2-6-linked sialic acid residues, with α1-6-fucose at the *N,N*'-diacetylchitobiose core. In the present study we concentrated on characterization of the oligosaccharide chains of purified native DARC glycoprotein from human erythrocytes.

Immunopurified Duffy glycoprotein was characterized in lectin blotting and by endoglycosidase digestion. More detailed studies were performed on DARC *N*-glycans isolated by in-gel PNGase F digestion. Brief analysis with lectins suggests that native Duffy *N*-glycans are mainly core-fucosylated (AAA, LCA, PSA) complex-type chains terminated with sialic acid residues (MAA, SNA), with bisecting β1-4 linked GlcNAc (PHA-E), similar to those of the recombinant form. Results of NP-HPLC analysis combined with exoglycosidase digestion indicate that DARC *N*-glycans are tri- and probably tetraantennary complex oligosaccharides with α1-6-fucose at the core, terminated with both α2-3- and α2-6-linked sialic acid residues.

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336: Structures and biosynthesis of the N- and O-glycans of recombinant human oviduct-specific glycoprotein expressed in human embryonic kidney cells (HEK293)

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Oviduct-specific glycoprotein (OGP), also known as oviductin, is a major mucin-like glycoprotein synthesized and secreted by non-ciliated secretory cells of mammalian oviducts. Oviduct-specific glycoprotein is conserved among all species examined to date including humans. The synthesis and secretion of OGP are under estrogen control. *In vitro* functional studies showed that OGP plays important roles during fertilization and early embryo development. We have recently produced recombinant human oviduct-specific glycoprotein (rhOGP) in human embryonic kidney 293 (HEK293) cells. The present study was undertaken to characterize the structures and determine the biosynthetic pathways of the N- and O-glycans of rhOGP. Treatment of the stable rhOGP-expressing HEK293 cells, respectively, with GalNAcα-Bn to block O-glycan extension, tunicamycin to block N-glycosylation, and neuraminidase to remove sialic acid residues increased the electrophoretic mobility of rhOGP. Detailed analyses of O- and N-linked glycans of rhOGP by mass spectrometry showed a broad range of simple and complex glycan structures. In order to identify the biosynthetic pathways involved in the glycosylation process of rhOGP, we assayed glycosyltransferase activities involved in the assembly of O- and N-glycans in HEK293 cells, and compared the results with those obtained from the immortalized human oviductal cells (OE-E6/E7). The latter OE-E6/E7 cells have previously been shown to retain the major characteristics of human primary oviductal cells and express mRNA transcripts and protein of human OGP. Our results demonstrate that both HEK293 and OE-E6/E7 cells exhibit activities of glycosyltransferases that synthesize elongated and sialylated O-glycans with core 1 and 2 structures as well as complex multiantennary N-glycans. It is anticipated that the knowledge gained from the present study will facilitate future studies of the role of human OGP and its glycans in fertilization and early embryo development. (Supported by grants from the Canadian Institutes of Health Research #MOP93606 to FWKK, the Canadian Cystic Fibrosis Foundation to IB, and the US NIH grant P41 RRO18502 to RO).

337: Drug-resistance inhibits expression of α2-6 sialylation on cell membrane glycoproteins

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Drug-resistance is one of the problems of cancer chemotherapy and a knowledge of the molecular changes associated with the development of resistance enables an understanding of the cellular mechanisms that are involved. We have focused on studying the changes in glycosylation that occur on membrane proteins of drug resistant T-cell acute lymphoblastic leukaemia (CEM) cells. The cells were selected for acquired drug-resistance against desoxyepothilone B (dEpoB), an antitumor drug that targets microtubules (CEM/dEpoB cell). Cell membrane fractions were obtained by Triton X114 detergent phase partitioning and ultracentrifugation. The glycomic profiles of the N-glycan and O-glycan alditols released from the cell membrane glycoproteins by sequential treatment with PNGase F and β-elimination respectively, were characterised by porous graphitized carbon LC-ESI MS/MS. The major N-glycan on CEM cells was the core fucosylated α2-6 monosialo-biantennary structure. Resistant CEM/dEpoB cells had a significant decrease of α2-6 linked sialic acid on all N-glycans. Moreover, this change occurred on all cell membrane glycoproteins. To understand the mechanism of this specific change in α2-6 sialylation on the proteins of cells with acquired resistance to dEpoB, we compared (1) protein expression level of β-galactoside α2-6-sialyltransferase (ST6Gal) and neuraminidase (NEU) by western blot, (2) enzymatic activity of ST6Gal and NEU by HPLC analysis using fluorescent pyridylamino-sugars as the substrates, (3) amount of donor substrate CMP-NeuAc by LC-ESI MS and (4) DNA sequence of ST6Gal, of CEM cells and CEM/dEpoB cells. These experiments at the gene, transcript and protein level determined that the lower α2-6 sialylation of the N-glycans on the cell membrane proteins of the drug resistant cells was caused by a decrease in activity of the enzyme ST6Gal, not by a loss of expression of the enzyme. We are now further investigating the relationship between α2-6-sialylation and drug-resistance.

338: ZIC-HILIC chromatography and ESI-QToF mass spectrometry for the compositional analysis and structural elucidation of complex heparin oligosaccharide mixtures

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Glycosaminoglycans (GAGs) are ubiquitous constituents of connecting tissue and cartilage where they are involved in many important biological processes such as blood anticoagulation, viral and bacterial infection, cancer and cell development. They are a class of complex glycans that form linear chains of up to 100 monosaccharide building blocks or more. The molecular structure of heparin comprises linear chains of repeating disaccharide units containing carboxyl and sulfate functional groups. The heparin backbone consists of 4GlcAβ1-4GlcNα1-subunits which can be N-sulfated/acetylated and O-sulfated at C2 position of the GlcA and at C6 position of the GlcN residue. Heparin is highly sulfated while the sulfation pattern is not consistent throughout the chain: highly sulfated regions alternate with sequences of a lower degree of sulfation. For their characterization GAG polymers are usually enzymatically digested to oligomers with a low degree of polymerization (dp) prior to mass spectrometric analysis. Since the resulting mixtures exhibit a high heterogeneity an efficient separaration is required to reduce sample complexity. Here, we show preliminary data on the effective chromatographic separation and mass spectrometric determination of GAG oligomer mixtures by use of zwitterionic hydrophilic interaction chromatography (ZIC-HILIC) coupled to a quadrupole time-of-flight mass spectrometer (QToF-MS).

The novel liquid chromatography MS-based approach enables the separation of heparin-derived di- and oligosaccharides according to their polarity and sulfation pattern. While chromatographic separation of disaccharides gives rise to discrimination of isomeric analyte species, mass spectrometric analysis allows the discrimination of co-eluting species. The utilization of the ZIC-HILIC approach for the analysis of higher oligomers also revealed a separation of isobaric species eluting in double peaks. MS^2 experiments were employed in this context to structurally characterize isomeric species for unambiguous assignment. The method presented in this contribution is highlighted as a rapid, efficient, and reliable technique to characterize heparin disaccharide composition as well as sulfated oligomers of different dp in complex mixtures.

339: N-glycosylation of the SKOV3 ovarian carcinoma cell line: cellular and secreted glycoproteins and secreted exosomes

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Ovarian carcinoma is the leading cause of death from gynecological cancers in many Western countries. Aberrant glycosylation is an important aspect in malignant transformation and consequently in ovarian cancer. In this study, structure analysis of the N-linked glycans of the SKOV3 ovarian carcinoma cell line has been performed using high-performance anion exchange chromatography with pulsed amperometric detection, MALDI-TOF mass spectrometry and lectin detection. N-Glycans from total cellular and secreted glycoproteins, as well as glycoproteins from the plasma membrane, microsomal fraction and secreted exosomes have been studied. Exosomes are membrane vesicles that are secreted by several cell types, including tumors. They contain several membrane and cytoplasmic proteins and, in cancer, they play a role in cell migration and metastases. Total cellular N-glycans consisted of high-mannose and proximally fucosylated complex partially agalactosylated structures (1). On the other hand, the LacdiNAc motif (GalNAcbeta4GlcNAc) was found in secreted glycoproteins as well as in plasma membrane and in exosome glycoproteins. Recombinant human EPO secreted from SKOV3 cells also contained the LacdiNAc motif and a large amount of N-acetylneuraminic acid in α2,3-linkage. Exosomes from SKOV3 cells contained specific glycoproteins that were distinctly recognized by the lectins Wisteria floribunda lectin (LacdiNAc), concanavalin A (binds α-mannosyl containing-branched glycans), Sambucus nigra lectin (recognizes NeuAcα2,6Gal/GalNAc) and Maackia amurensis lectin (NeuAcα2,3Galβ1,4GlcNAc/Glc) (2). These may constitute exosome markers. The N-glycosylation profiles of exosomes have also been investigated. This work contributes to the knowledge of the glycosylation of a human ovarian cancer cell line. Furthermore, the knowledge of exosomes glycosylation with the possibility for its modulation will open new perspectives in cancer vaccination. References: (1) Machado, E., Kandzia, S., Carilho, R., Altevogt, P., Conradt, H.S., Costa, J. (2011) Glycobiology 21, 376 (2) Escrevente, C., Keller, S., Altevogt, P., Costa, J. (2011) BMC Can.00, 000.

340: Glycosylation profile of integrin alpha3beta1 subunits in human melanoma cells at different stages of progression

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In recent years the glycobiological studies seems to get out of a niche and become to be treated supplementary to proteomics. It is well known, that protein N-glycosylation changes upon neoplastic transformation. These changes are potentially different in case of primary and metastatic cells, and may influence their behavior. The detailed structural studies on metastasis-involved glycoproteins will be undoubtedly very helpful in understanding the information "hidden" behind cancer-related glycosylation changes. The research model of this work consists of the pair of human melanoma cell lines (primary MW115 and metastatic WM266-4) derived from the same patient and thus representing different stages of disease progression. The integrin alpha3beta1 was isolated using affinity chromatography. After PNGaseF treatment, released N-glycans were fluorescently labelled and analysed by NP-HPLC. The glycosylation of total cell proteins, precipitated from whole-cell lysates was also studied, as a glycomic background. The glycan profile of total proteins from both cell lines were very similar. The majority of glycans were high mannose structures (Man5, Man6, Man7, Man8 and Man9). The relative amount of charged species, mostly sialylated, was quite small. The both cell lines displayed also very similar profile of integrin alpha3beta1 glycosylation. In contrast to total cell protein derived glycans, the charged species (mostly monosialylated di- or triantennary comlex type glycans) were in the majority.

The results of our studies suggest, that in some cases the primary and metastatic cells do not differ significantly in protein N-glycosylation. It is still not clear whether the differences in integrin glycosylation between primary and metastatic melanoma cell lines observed so far result from the changed activity of glycosylation machinery at the level of primary tumor formation, specific for individual case or are a dynamic process accompanying each stage of melanoma progression. Futher research, utilizing diverse models is needed. This work was supported by the polish MNiSzW grant no. N N301 304637

341: Novel O-linked glycans containing 6'sulfo-Gal/GalNAc of MUC1 secreted from human breast cancer YMBS cells

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MUC1 is often secreted in the blood stream from the early stages of invasive and metastatic cancers including breast, stomach, pancreas, colon etc. Since the structures of O-linked glycans change by tumorigenesis, we focused on their structural studies. MUC1 secreted from human breast cancer was purified from human breast cancer YMB-S cells by using MUC1-specific KL-6mAb affinity column chromatography. O-Linked glycans were liberated from MUC1 by hydrazinolysis and reduced with NaB^3H_4. Released ^{3}H-glycans were separated into four acidic fractions with pH 5.4 paper electrophresis. Their structures were determined in combination with sialidase digestion, methanolysis, Bio-Gel P-4 column chromatography, several lectin column chromatography including RCA-I, TJA-I, MAL, PVL, Galectin-3, and SNA. The determined structures are as follows. Neu5Acα2-3(±SO_3^--6)Galβ1-4GlcNAcβ1-3Galβ1-3GalNAc, Neu5Acα2-3Galβ1-4(±SO_3^--6)GlcNAcβ1-3Galβ1-3GalNAc, SO_3^--3Galβ1-4(±SO_3^--6)GlcNAcβ1-3Galβ1-3GalNAc, Neu5Acα2-8Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-3GalNAc, ±Neu5Acα2-3Galβ1-3(±NeuAcα2-6)GalNAc, ±SO_3^--3Galβ1-3(±SO_3^--6) GalNAc. The ratio of core1 and extended core1 were 48:52, and the ratio of sialic acid, sulfate, and sialic acid/sulfate containing glycans were 41:42:17. These structures of sulfated O-linked glycans were unique in comparison with previously reported structures from several breast cancer cell lines. Futhermore, based on the identified structues, synthetic glycans binding to anti-KL-6mAb were compared with one another by surface plasmon resonance method, and related radiolabeled oligosaccharides were enzymatically synthesized and analyzed for their binding to an anti-KL-6 mAb-conjugated affinity column. As the results, 3'-sialylated, 6'-sulfated extended core1[Neu5Acα2-3(SO_3^--6)Galβ1-4GlcNAcβ1-3Galβ1-3GalNAc], 3'-sialylated, 6-sulfated core 1[Neu5Acα2-3Galβ1-3(SO_3^--6)GalNAc], and di-sulfated core1 SO_3^--6 (SO_3^--3Galβ1-3GalNAc) exhibited substantial affinity for the anti-KL-6mAb, and 3'-sulfated core1 derivatives [SO_3^--3Galβ1-3(±Neu5Acα2-6) GalNAc] also interacted with KL-6mAb. These unique multiple carbohydrate epitopes of KL-6mAb may be related to the multi-reactivities for not only interstitial pneumonia but also lung, liver, breast, pancreas, stomach and colorectal cancers. It is interesting which sulfotransferases including Gal6ST and Gal3STs2-4 increase in the respective cancer tissues in relation to their metastatic abilities. We are now investigating whether these sulfotransefeses change in patients with invasive/metastatic breast cancers.

342: Cell Surface Glycan Analysis Platform

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We introduce a glycomics platform for analyzing cell surface glycans. Cellular glycans are generally analyzed from a total cell lysate, although the glycans involved in cellular communication reside on the cell surface. Therefore the novel workflow begins with specific and cell type optimized isolation of cell surface proteins. Glycans are released from cell surface proteins and purified by miniaturized solid-phase extraction. Glycans are reduced to eliminate anomer peaks and permethylated when necessary prior to analysis by LC-MS^n. Novel MS data analysis software, developed in house, is applied to simplify the data handling. The glycan LC-MS/MSMS identification workflow combines existing proteomic software and novel glycan specific tools based on the in house developed R library called GlycanID. The novel LC-MS based glycan analysis platform is especially suitable for comparative analysis between samples. For example it can be used to elucidate differences in the glycosylation of functionally separate cell populations. We focus on cells with therapeutic potential. Lately we have analyzed glycosylation of multiple stem cell types, such as hematopoietic stem cells, and mesenchymal stem cells with immunosuppressive potential. Also other immunological cells and intestinal microbes are being analyzed.

343: Combined preparative sodium deoxycholate polyacrylamide slab gel electrophoresis, reverse staining and passive elution techniques: Applicability to the separation and biochemical analysis of intact bacterial lipopolysaccharide species by using mass spectrometry and in vitro bioactivity assays

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A methodology for the fractionation of bacterial lipopolysaccharides (LPS) by preparative SDS- or DOC-slab-PAGE, reverse staining and passive elution in 5% triethylamine (TEA) has been recently established. Here, the suitability of this method for separating and analyzing intact LPS species by using ESI FT-ICR mass spectrometry (MS) and *in-vitro* bioactivity assays is evaluated. First, the chemical integrity of isolated LPS fractions was tested by ESI FT-ICR MS. The degradation of LPS in 5% TEA consisting of the cleavage of alkaline-labile ester-linked fatty acids could be prevented by selecting a relatively short (5 min) incubation time and neutralizing this basic solvent afterwards with acetic acid. The elution of the Re LPS of *E. coli* F515, or the rough and semi-rough LPS species of smooth-type *E. coli* O111 from gels under these incubation conditions resulted in up to milligram quantities of electrophoretically homogeneous LPS fractions without any appreciable alteration in lipid A integrity. Exhaustive desalting of LPS fractions prior to ESI FT-ICR MS by dialysis followed by ethanol-acetone precipitation made it possible to obtain high-quality MS spectra of unmodified LPS species reproducibly. Purified LPS fractions of *E. coli* O111 remained highly active in inducing the production of TNF-alpha in human macrophages and IL-8 in TLR4/MD-2-transfected human embryonic kidney (HEK293) cells *in-vitro*. On the other hand, they were notably less active than the initial unfractionated LPS in inducing the production of IL-8 in TLR2-transfected HEK293 cells, consequently demonstrating that TLR2-activating contaminants present in the initial LPS preparation were efficiently removed by the isolation procedure. Therefore, this methodology proved suitable for fractionating and biochemically analyzing intact molecular species of LPS by using the aforementioned analytical methods.

344: High-Throughput Characterization of the Human Milk Oligosaccharide Composition Utilizing a Multiplexing Capillary DNA Sequencer

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During the last decade an enormous progress regarding knowledge about the composition and properties of human milk (HM) has been made. The three main fractions of HM are proteins, fats and carbohydrates. They combine a large variety of properties and functions, like lowering the risk of infections or balance/teach the developing immune system of newborns. The project presented describes the establishment and improvement of a glycoanalysis technique, based on multiplexed capillary gel electrophoresis with laser induced fluorescence detection (CGE-LIF), which allows for high-throughput (HTP) characterization of human milk oligosaccharides (HMOS). The HMOS fraction primarily consists of lactose and a large variety of neutral and acidic oligosaccharides. The characterization of HMOS is essential to understand the relationship between structure and biological effect. Therefore, a rapid and sensitive HTP-method for the analysis of the HMOS fraction composition was established. The method comprises protein-precipitation, clean-up, derivatization as well as the analysis by multiplexing CGE-LIF, using a "ABI PRISM 3100 Genetic Analyzer™ ". In combination with an in-house HMOS database, the generated HMOS "fingerprints" (normalized electropherograms) can be used for in-depth analysis, i.e.: structural elucidation of each single compound and its relative quantification. The method developed was tested on various HM samples (time series of different donors). Several factors were identified to be responsible for differences in HMOS-composition, including origin of the

mother, lactation time point and genetic determinants (secretor-gene (Se) and lewis-gene (Le)). The combination of these two antigenic determinants defines four human milk types [1]. It turned out, that multiplexed CGE-LIF is a valuable alternative to the existing time consuming analysis techniques. The method and system developed [2], allows a straightforward, fast, unequivocal and sensitive analysis of HMOS fraction composition including an easy determination of secretor and lewis status of milk donors.

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345: A Software Tool for Automated High-Throughput Processing of CGE-LIF Based Glycoanalysis Data, Generated by a Multiplexing Capillary DNA Sequencer

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Glycomics is a rapidly emerging field that can be viewed as a continuation of the genomic and proteomic era. Hence, there is a dynamic increase in the demand for sophisticated databases and smart analytical tools in glycobiology, respectively, glycobiotechnology. In order to enhance and improve the comparatively small existing glycoanalytical toolbox a fully automated (high-throughput (HTP) and high-resolution (HR)) analysis method with a fully automated data evaluation is required. Besides several mass spectrometry and liquid chromatography based analysis techniques, electromigrative separation techniques for the analysis of oligosaccharides became apparent during the recent past. Especially, capillary gel electrophoresis with laser induced fluorescence detection (CGE-LIF) as one feasible electromigrative separation technique - using standard DNA sequencer equipment - has been established for HTP glycoprofiling of APTS-labeled glycans [1]. The application of this technique using instruments with up to 96 capillaries in parallel to glycoanalysis, results in massive reduction of the effective separation time per sample combined with an impressive sensitivity achieved due to LIF detection [2].

Due to the lack of appropriated software for data analysis, the MATLAB® based software tool "glyXtool" was developed, now significantly reducing the all-over analysis time per sample. A graphical user interface makes this tailor made glycoanalysis software-tool for automated data-processing easy to handle also by non-experts. Thereby, glyXtool provides automated background adjustment, raw data smoothing, migration time normalization to an internal standard, peak picking and peak integration in HTP. All this, combined with glyXtools intrinsic sample comparison function, a fully automated peak annotation and an interface to a continuously growing oligosaccharide database, makes multiplexed CGE-LIF to a powerful glycoanalysis tool. This is in contrast to currently prevailing methods, where multiplexing with respect to HTP is highly cost and lab-space intensive and ties up a lot of manpower and experts hands-on-time.

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346: High-Throughput CGE-LIF Based Analysis of APTS-labeled N-Glycans, Utilizing a Multiplex Capillary DNA Sequencer

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The analysis of protein glycosylation became of increasing importance in research, as well as for monitoring production processes of biotechnologically produced pharmaceuticals, in the recent past. Applications in both fields require high-throughput (HTP) and high-resolution (HR) analytical methods that allow fast and robust profiling of protein glycosylation on large sample sets. To characterize these complex posttranslational modifications of proteins several analysis methods were developed in the last three decades. Besides several mass spectrometry and liquid chromatography based analysis techniques, electromigrative separation techniques became apparent during the recent past. Especially capillary gel-electrophoresis with laser-induced-fluorescence-detection (CGE-LIF), using standard DNA sequencing equipment, has been developed for HTP glycoprofiling of APTS-labeled glycans [1]. The application of this technique with up to 96 capillaries in parallel, results in massive reduction of the effective separation time per sample combined with an impressive sensitivity due to LIF detection. Here we present an optimized sample preparation procedure for N-glycan-profiling by CGE-LIF, enabling the HTP glycosylation analysis of e.g. human plasma N-glycome [2]. After enzymatic glycan-release, N-glycan labeling with APTS was performed, using 2-picoline borane as a non-toxic reducing agent. Reaction conditions were optimized for a high labeling efficiency, short handling times and only limited loss of sialic acids. A polyacrylamide

based stationary phase was used in hydrophilic-interaction-chromatography (HILIC) mode to purify APTS labeled glycans. The whole sample preparation procedure can be performed at the 96-well-plate level with a hands-on time of less than 2.5 h. Purified APTS-labeled N-glycans were analyzed using a standard capillary DNA sequencer. The optimized sample preparation, combined with robust, HR, high-sensitive multiplexed CGE-LIF based measurement and fully automated data evaluation, enables highly reproducible "real" HTP analysis of protein N-glycosylation.

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347: Tailored methods for CGE-LIF based multiplexed high-throughput glycoanalysis with respect to sample characteristics

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Glycomics is a rapidly emerging field, that can be viewed as a complement to other „omics" approaches including proteomics and genomics. Hence, there is a dramatic increase in the demand for sophisticated databases and analytical tools in glycobiology, respectively glycobiotechnology. In order to enhance and improve the comparatively small set of existing glyco-analytical methods and toolboxes, a system and method for automated straightforward, sensitive high-throughput (HTP) and high-resolution glycoanalysis was developed [1,2]. This glycoanalysis approach is based on multiplexed capillary gelelectrophoresis with laser induced fluorescence detection (CGE-LIF), using a capillary DNA-Sequencer (Applied Biosystems). Data is evaluated in conjunction with a novel modular software-tool for data-processing and automated structural elucidation by interfacing a corresponding oligosaccharide-database. The aim of the project presented was to develop tailor-made workflows for multiplexed CGE-LIF based glycoanalysis, optimized for different types of samples, each with its specific characteristics. Exemplarily, three different sample types were chosen: first, a broad variety of N-glycans released from citrate plasma proteins (a complex sample of medium to large, charged and uncharged glycostructures); second, a relative small set of N-glycans released from IgG (a modest sample with medium sized mainly uncharged glycostructures); and third, the complete pool of oligosaccharides from human breast milk (complex sample of small charged and uncharged glycostuctures). The whole workflow starting from sample clean-up until CGE-LIF analysis using a standard capillary DNA Sequencer was optimized individually to each of the three representative samples. For sample preparation, different chromatographic techniques and materials were tested and different polymers and capillary lengths were investigated for final CGE-LIF analysis.

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348: Trypsin is not enough - Glycoproteomic analysis of recombinantly expressed proteins by mass spectrometric approaches

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For the efficient and safe use of glycoprotein pharmaceuticals, it is necessary to analyse the structures of the carbohydrate moieties of those glycosylated drugs. Analysis of the consistency of protein glycosylation at the glycopeptide level allows the use of standard proteomic methods, which take advantage of the favourable ionisation properties of the peptide moiety that exceeds the lower proton affinity of sugars. As a consequence, the glycopeptide spectra approximate the true glycosylation pattern at a given site. For this reason we used this approach to analyse the glycosylation status of the recombinantly expressed glycoprotein drugs human erythropoietin (r-hEPO) and human follicle stimulating hormone (r-hFSH). Trypsin digest of r-hEPO resulted in one O-glycopeptide and two N-glycopeptides, one of them comprising two glycosylation sites. Since each of those sites carried various glycan forms, the obtained spectra were highly complex. An additional digest with endoproteinase GluC led to clear spectra of the separated glycosylation sites. In the case of r-hFSH, only three of the four expected tryptic glycopeptides were found, whereas after chymotryptic digestion, all four glycopeptides could be identified. Three sites were occupied by mainly di-antennary N-glycans, whereas the glycopeptide three on the FSH-unique ß-chain carried larger N-glycans with lactosamine repeats. Glycopeptides were finally quantified by the software MassMap®, which determines the intensity of an entire mass peak including the relevant isotopes, adducts and different charge states. Thus, a reproducible quantification of glycoforms in complex data sets can be achieved. The glycopeptide data were corroborated by total oligosaccharide analysis performed by LC on porous graphitic carbon with mass spectrometric detection.

349: Comparing Sialic Acid Determination in Glycoproteins: Two Liquid Chromatography Methods

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Sialic acids are critical in determining glycoprotein bioavailability, function, stability, and eventual catabolism. Although over 50 natural sialic acids have been identified, two forms are commonly determined in glycoprotein products: N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Because humans do not generally synthesize Neu5Gc and have been shown to possess antibodies against Neu5Gc, the presence of this sialic acid in a therapeutic agent can potentially lead to an immune response. Consequently, glycoprotein sialylation, and the identity of the sialic acids, play important roles in therapeutic protein efficacy, pharmacokinetics, and potential immunogenicity. This work compares two chromatographic sialic acid assays. Analyses by both high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) and UHPLC with fluorescence detection (UHPLC-FLD) are evaluated with comparisons of assay performance and total analysis time. Calibration ranges were chosen spanning the expected amounts of Neu5Ac and Neu5Gc in five representative glycoproteins; calf fetuin, bovine apo-transferrin, human transferrin, sheep α_1-acid glycoprotein, and human α_1-acid glycoprotein. For both methods, response is linear with correlation coefficients >0.9990 and >0.995 for HPAE-PAD and UHPLC-FLD determination, respectively. Glycoprotein hydrolysates were analyzed to evaluate method accuracy and precision. In both cases, method sensitivity easily allows detection of the sialic acids in prepared acid hydrolyzates. Retention time precision (RSDs of <0.12) and peak area precision (RSDs of <3.0) are good for both methods. Both methods investigated provided results that were consistent with previously published results for the glycoproteins that were analyzed. Additionally, recoveries ranged from 79-114% for Neu5Ac and 81-110% for Neu5Gc by UHPLC-FLD and from 74-107% and 72-103% for Neu5Ac and Neu5Gc by HPAE-PAD, respectively, suggesting method accuracy. Between-day assay precision (RSDs) ranged from 9.5-19% (UHPLC-FLD) and 7.9-14% (HPAE-PAD). Results from the example glycoproteins are used to illustrate method precautions and advantages.

350: Fast Sialic Acid Determination of Glycoproteins by High Performance Anion Exchange – Pulsed Amperometric Detection

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Due to their critical role in protein chemistry, sialic acids are commonly determined in protein therapeutic products. These carbohydrates are important in controlling glycoprotein bioavailability, function, stability, and metabolism. Although over 50 natural sialic acids have been identified, two forms are commonly determined in glycoprotein products: N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Determination of these two sialic acids is frequently performed in two steps; acid hydrolysis is typically used to release the sialic acids, which is then followed by a chromatographic method to determine the amounts of Neu5Ac and Neu5Gc and therefore the total sialic acid content. Among the chromatographic methods for determination, there are direct detection methods, such as High Performance Anion Exchange-Pulsed Amperometric Detection (HPAE-PAD), and those that require further sample derivatization for analyte detection, such as fluorescent labeling followed by UHPLC. This work describes the development of an assay for glycoprotein sialic acids with an acetic acid hydrolysis followed by direct and fast (5 min) analyte determination by HPAE-PAD. Calibration ranges were chosen spanning the expected amounts of Neu5Ac and Neu5Gc in five representative glycoproteins. Response is linear between 0.27-68 pmol for Neu5Ac and 0.23-11 pmol for Neu5Gc with correlation coefficients ^{3}0.9995. The method sensitivity easily allows detection of the sialic acids in diluted hydrolyzates, reducing the need for lyophilization prior to sample analysis. Retention time precisions (RSD of <0.8 for both sialic acids) and peak area precisions (RSDs of <1.98 for Neu5Ac and Neu5Gc) for standards are excellent. Recoveries from glycoprotein hydrolyzate samples ranged from 81-96% for Neu5Ac and 82-106% for Neu5Gc, suggesting accuracy. This 5 minute assay allows the rapid, direct, and accurate quantification of sialic acids in glycoprotein hydrolyzates, providing a convenient method for quick screening of expression optimization experiments without the costly and time consuming derivatization steps required for existing UHPLC fluorescence detection methods.

351: Total glycomics as cellular/tissue descriptors

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A number of carbohydrate-related biomarkers have been reported to possibly correspond to distinct cellular states such as differentiation, proliferation, or to cell types. Evaluation of cellular states based on total glycomics which focuses on all major classes of complex carbohydrates (i.e., glycoproteins, glycolipids, glycosaminoglycans) would further accelerate the progress. Using chemoselective glycoblotting technique [1] as a core technique for the purification of glycans from

crude biological mixture, we established novel analytical procedures not only for N-glycans but also for glycans of glycosphingolipids and glycosaminoglycans. The established method was first validated by analyzing cellular glycome of various model cultured cell lines (e.g. CHO-K1 and its lectin-resistant mutants, K562, NIH/3T3, HL60, etc). Upon confirming its validity to analyze quantitative total cellular glycomics, the methods were further applied to the various ocular tissues, including cornea, limbus, and conjunctiva, which revealed distinct tissue specific glycomic profiles. Glycomic profile of epithelial cell sheet for corneal regeneration fabricated on temperature-responsive culture surfaces from oral mucosal epithelial cells [2] will be also discussed.

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352: Structural elucidation of the glycosylation of *Francisella tularensis* immunoreactive proteins using a nanoLC-ESI-FT-ICR mass spectrometer

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Background: The glycosylation is among the most common post-translational modifications, having an influence on the protein properties and playing a crucial role in many biological processes. It has been found that glycoproteins of many pathogenic bacteria are associated with virulence factors. Recently, the presence of glycoproteins has been studied in a highly infectious Gram-negative bacterium *Francisella tularensis*. A wide spectrum of putative *F. tularensis* glycoproteins was characterized through the combination of carbohydrate-specific detection and enrichment techniques with highly sensitive mass spectrometry. The studies pointed out 12 proteins, which represent the best candidates for *F. tularensis* glycosylation.

Methods: The membrane protein-enriched fraction of the *F. tularensis* subsp. *holarctica* strain FSC200 was separated using 2D gel electrophoresis followed by carbohydrate-specific staining. In-gel tryptic digests of selected putative glycoproteins were analyzed using nanoHPLC on-line coupled with LTQ-FT-ICR mass spectrometer. Samples were desalted and preconcentrated on a C18 μ-precolumn. The separation of peptides was conducted at a flow rate of 250 nL/min using a C18 analytical column with a linear gradient, from 3 to 55 % phase B (3/97/0.1, water/ACN/FA, v/v/v) over a period of 45 min and ramped from 55 to 80 % ACN over 10 min. MS spectra were acquired in the mass range from 80 to 2,000 m/z followed by the fragmentation of five the most intense peaks. In order to acquire information on the glycan(s) and particular glycopeptides, a survey scan was defined in an instrument method. In this scan event, diagnostic glycan oxonium ions were generated by in-source CID at an orifice potential 100 V. Results: On the basis of previous results, several putative glycoproteins were selected for a detail examination on glycosylation. The proteins were analyzed using nanoHPLC-ESI-FT-ICR using a stepped-orifice voltage-scanning. Manual inspection of the acquired data led to the structural characterization of an identical glycan structure modifying investigated proteins and the identification of glycan-modified peptides. The glycan was determined to be a hexasaccharide consisting of hexoses, N-acetylhexosamines and an unknown monosaccharide. Further definition of the proteins modified by glycan(s) is needed, faciliting a better understanding of the possible function of protein glycosylation in pathogenicity of the microbe. Acknowledgments: This work was financially supported by Ministry of Education No. MSMT0021627502 and No. ME08105, Ministry of Defence, Czech Republic No. FVZ0000604, and Czech Science Foundation No. GA203/09/0857.

353: Robustness testing of the high throughput HPLC-based analysis of plasma N-glycans

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Analysis of plasma protein glycosylation by high throughput HPLC-based 96 well platforms is becoming a routine tool for the analysis of large sample sets in studies comprising thousands of individuals. Analytical method which is routinely used to obtain a large amount of data should be well characterized and all potentially critical steps should be identified. Robustness of the high throughput method was tested by Plackett Burman two level, 11-factor, 12 experiment screening design. For initial screening eleven potentially important factors were chosen. The response variable was calculated as a coefficient of variance between area % of each peak in the each reaction and the area % obtained after performing the procedure according to the laboratory standard operating procedure with standardized plasma samples. Thirteen out of 16 glycan groups separated by HPLC revealed statistically significant changes with varying response to 11 factors, which was expected due to their structural and chemical differences. Time of exposure to DTT (DTT), Time of storage prior to formic acid exposure (STORE1), time of exposure to formic acid (FORMIC), duration of labeling reaction (2AB) were identified as the most critical steps which should be closely monitored to avoid introduction of analytical errors in the analysis.

354: Multiplex labelling with aniline and 2-aminobenzamide enable parallel chromatography analyses of different N-glycan samples

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The complexity of branched glycan structures composed of similar monomeric units hinders their analysis and the knowledge about biological functions of glycans is lagging far behind the knowledge about other macromolecules. HPLC is one of the principal analytical tools for glycan analysis, but complex biological samples require long runs that limit analytical capacity. One of the most widely used fluorescent labels for glycans is 2-aminobenzamide. Here we show that glycans labelled with aniline and 2-aminobenzamide can be simultaneously analyzed on hydrophilic interaction columns since they have non-overlapping excitation and emission spectra, and the attached labels have very similar influence on the retention times of glycans. This method can make glycan analyses faster and also enable introduction of internal standards.

355: Synthesis of derivatives of biologically important carbohydrates and their incorporation into microarrays

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Glycoconjugates, such as glycoproteins and glycolipids, are major components of all cell membranes and as such they are crucial in cell signalling, cell recognition and other cell-cell interactions in many physiological and pathological processes [1]. The aim of this project is to synthesise a range of sialic acid derivatives, to incorporate them into trisaccharides to form unnatural sialosides using both chemical and chemoenzymatic methods, and to study the sialosides' binding behaviour towards proteins in microarrays. Synthesis of the sialic acid derivatives involved two complementary methods. Thus derivatisation either involved chemical manipulation of sialic acid itself, or conversion of mannose or N-acetyl mannosamine derivatives to sialic acid-nucleotides using chemoenzymatic protocols [2]. In the former case halogenation, oxidation and acetylation reactions were probed. In the latter cases, halogenation and acetylation reactions were investigated. The obtained sialic acid derivatives will be transferred onto disaccharide acceptors, such as lactose, N-acetyl lactosamine or S-linked disaccharide mimics, using sialyltransferases, to give unnatural sialosides. These will then be incorporated into microarrays to probe their interaction with lectins, in particular with selectins and siglecs [3]. Findings in this area will be very useful for the development of new therapeutic options, e.g. for the treatment of cancer and associated processes like tumour-cell adhesion and metastasis, or for the modulation of immune cells in immune-cell-mediated diseases.

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356: Understanding N-glycan-protein interactions through binding assays on glycoarrays

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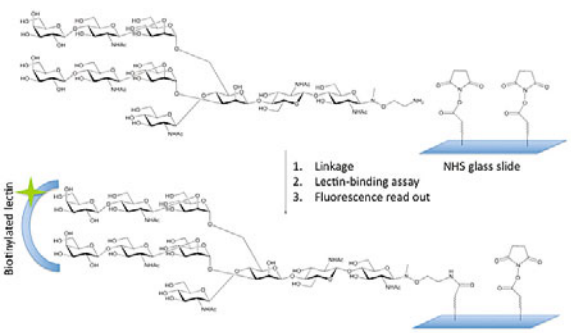
Specific interactions between carbohydrates and proteins are often essential in viral and bacterial infections, immune response and development of tumor cell metastasis. A typical class of oligosaccharides involved in those processes are N-glycans. Of special interest are bisected N-glycans, since they are correlated to cancer [1] and can be used to isolate stem cells [2]. Due to the structural variability of carbohydrates the efficient isolation of homogenous glycans from natural sources is strongly hampered. Thus the investigation of the functions of glycosylation patterns was addressed by a synthetic approach. We developed an efficient pathway combining chemical [3] and enzymatic [4] synthesis in order to obtain complex type bisected carbohydrates. Through synthesis we obtained a complete library of 16 bisected N-glycans, which varied in the number of antennae, their branching mode and the terminal sugar residues. After the attachment of a bifunctional linker [5] the library was printed on N-hydroxysuccinimide activated glass slides. Within this microarray platform the printed carbohydrates were tested with several plant lectins. The further elaboration of this microarray strategy should lead to a better definition of the specificity of carbohydrate-protein interactions in general.



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357: Fucosyltransferases as Synthetic Tools: Matching Substrate and Enzyme with the Help of N-glycan arrays.

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Core-fucosylation is one of the most abundant modifications of asparagine-linked glycans of many eukaryotes [1]. Core α-1,6 fucose is a conserved epitope presented in both invertebrates and vertebrates. In mammals, it is the unique fucosylation in the chitobiose core of N-glycans and differences in the expression of this epitope in glycoproteins have been studied as biomarkers for cancer [2]. In invertebrates and plants core α-1,3 fucosylation is prominent and is suspected to be an antigen for IgE mediated allergy [3]. The development of efficient screening tools of the specificity of fucosyltransferases could be useful for the characterization of enzymes and for their application in the synthesis of therapeutic proteins and in the preparation of glycoconjugates vaccines. By combining modular synthesis of core structures with on-chip enzymatic elongation we have recently prepared a microarray focused on synthetic N-glycans [4]. In the present work, this N-glycan microarray platform finds application in determining the substrate specificity of different core type α-1,3 and α-1,6-fucosyltransferases from *Arabidopsis thaliana* and *Caenorhabditis elegans* expressed in the yeast *Pichia pastoris* [5]. A comparative study of different fucose binding lectins (*Lens culinaris* agglutinin, *Pisum sativum* agglutinin, *Aspergillus oryzae*, *Aleuria aurantia* and anti-horseradish peroxidase antibody) was also performed on the array after the enzymatic elongation. Once substrate specificities of glycosyltransferases were established, we applied selected fucosyltransferases as synthetic tools for the preparation of fucosylated N-glycans on a preparative scale.

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358: N-glycosylated Glycoproteins characterized by Mass Spectrometry – an Integrated Software Approach

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In biochemistry, glycosylation is of increasing interest. Protein glycosylation, which is a key post-translational modification, is important e.g. for protein folding and for cell to cell adhesion. To study the biochemical function, a detailed structural characterization is required, and mass spectrometry is a highly suitable technique for this purpose. However, the analysis of the N-glycosylation pattern present on glycoproteins is challenging due to the heterogeneous glycan structures and ion suppression effects. For the interpretation of glycopeptide MS/MS spectra via database search, the knowledge of the peptide and glycan mass is required. This can be automatically handled in the presented software approach. To characterize the N-glycosylation patterns, a bottom-up approach was chosen. Tryptically digested glycoprotein samples were separated by nano LC and analyzed by ESI and MALDI mass spectrometry. All MS/MS spectra, which have been recognized as originating from glycopeptides, were supplied to protein and glycan database searches in SwissProt and GlycomeDB, respectively. The analysis of a digested monoclonal antibody provided, in addition to a nearly complete sequence coverage of the non-glycosylated peptides, a detail-rich picture of the highly complex pattern of N-linked glycans. 28 potential glycopeptide spectra resulted. 24 of them could be unambiguously identified resulting in two glycopeptide sequences and 15 different glycan compositions. For alpha-1-acid-glycoprotein, all 5 glycosylation sites were identified, partly as highly sialated glycans. These examples demonstrated fully automatic identification of glycopeptides and detailed characterization of the glycosylation pattern, profiting from the highly interactive result validation in our integrated software solution. This facilitates the initial characterization of the glycoproteins as well as the subsequent quality control tasks, e.g. for biotherapeutic proteins.

359: A bioinformatics tool for the prediction of changes in glycosylation of cells after exposure to various stress conditions.

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Glycosylation is one of the most important forms of protein modification and plays an essential role in biological

processes. Alterations of carbohydrate structures reflect small variation in the cellular environment. Thus, glycan antigens can be used for diagnosis and therapy response monitoring. Glycosylation is a very complex process regulated by a number of factors. Additionally, only a small amount of glycans can be found in a biological sample, which is one of the main difficulties of carbohydrate structure analysis. In this work we present a novel application which incorporates the most up-to-date public glycan structure repository along with an experimentally validated glycotransferases database in order to predict a set of glycan chains with the highest probability of occurrence in the conditions analyzed, based on the transcriptome state. This application was used to determine changes in glycosylation patterns of several radiation-treated cancer cell lines, and showed a reasonable agreement of the predicted glycan sets with changes in the cell surface glycans detected using fluorescent lectins. This work shows that by combining modern transcriptomic analysis methods with data gathered in glycan-oriented databases through the last quarter of a century, it is possible to significantly increase the possibilities of glycan-based cell response studies. This work was supported by Silesian University of Technology and by grants PBZ/MEiN/01/2006/49, N N518 283 640 and N N518 497 639 from the National Science Centre.

360: The use of a novel epitope library for the annotation of glycan structures

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The development of the UniCarb-DB (www.UniCarb-DB.org) database of MS/MS spectra of glycan structures, as part of the UniCarbKB KnowledgeBase, puts glycomics firmly in the realm of frontier science today. However the resource is only useful as long as it is being updated, in both a quantitative and qualitative manner. Quantitatively the UniCarb-DB database contains detailed MS data on glycan structures that originate from healthy/control type samples and from multiple disease states. Added to this is the inclusion in the database of information on glycans on the same glycoprotein from different tissues. From previous studies we have seen that the main difference between tissue dependent expression of glycans (from, for example, mucins) is found in the terminal epitopes that are carried by the core structures. The most recent qualitative addition to the initiative is the inclusion of a glycan epitope library. This library defines oligosaccharide sub-structures as a "functional unit" and the population of O-linked glycan structures in UniCarb-DB has been annotated to reflect this. The database allows the user to query one, or many, glycan structures based on the presence of these epitopes. One function of this glycan epitope library is the identification of relationships between glycan structures based on their containing similar sub-units. The availability of a resource such as this will allow biological questions based on the functionality of these sugar epitopes to be more easily addressed, with the differences and/or similarities between oligosaccharides on proteins available at the click of a button.

361: Structural features and immunological activity of N-glycans of seaweed glycoproteins

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N-Glycans linked to plant glycoproteins have some unique structural features, the combinational occurrence of α1-3Fuc and β1-2 Xyl residues on the trimannosyl core and Lewis a epitope at the non-reducing end. The former unit has a strong immunological activity against mammals and can be antigenic epitope that IgG and IgE recognize. These structural features of N-glycans of terrestrial plant have been characterized well but those of hydrophytes remain to be elucidated. In this study, therefore, we analyzed the structures of N-glycans linked to seaweed glycoproteins. First, glycopeptides were prepared from the pepsin-digests of seaweed homogenates (brown algae, red seaweeds, and green laver) by gelfiltration and IE-HPLC. Glycan moieties were liberated by hydrazinolysis and then were fluorescence-labeled. The structural analysis using HPLC-mapping showed that major N-glycans of hydrophytes are typical high-mannose type structures, while the typical plant complex type N-glycans having α1-3Fuc and β1-2 Xyl residues were not found. However, we found some unique glycans that were resistant to several glycosidases (α-Man'ase, β-HexNac'ase, β-Gal'ase, and α-Fuc'ase), indicating that some hydrophytes-specific glycans might occur on some seaweed glycoproteins. Furthermore, the immunological activity of the seaweed glycopeptides was assayed, and it was found that some glycopeptide-fractions could stimulate human monocytes.

362: Gastropod glycan elucidation with focus on rare sugar constituents

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One of the most commonly used methods for glycan elucidation apart from MS analysis is HPLC analysis of

fluorescently labeled glycans before and after digestion with glycosidases. Due to the specificity of these enzymes, accurate information about glycan composition and structures is gained. Although this is a reliable method for common glycans, we have encountered dramatic limitations in snail glycan analysis. We have shown that this is a fact of methylated sugar compounds which protect the snail glycans from digestion. While methylation is a rather rare glycan modification, our monosaccharide analyses on HPLC and GC have confirmed the occurrence of 3- and 4-O-methylated galactose as well as 3-O-methyl-mannose in mollusc glycans in agreement with literature data. Apart from these unusual modifications several difficulties arise during snail glycan analysis due to the tough tissue and the rigid mucus. The removal of storage carbohydrates which might influence monosaccharide analysis and the separation of N- and O-glycans are other stuggles to resolve. While the first was done by α-glucosidase digestion from rice, the latter is an even more difficult task. While N-glycosidases have their limitations and do not completely remove N-glycans, even mild β-elimination conditions seem to be sufficient to cleave some N-glycans of the peptide backbone which gives N-glycan impurities in O-glycan fractions. Therefore MS structure analysis and the knowledge of biosynthetic pathways have to be consulted to gain more insight into the fascinating glycosylation pattern of gastropods. The combination of ESI-MS as well as MALDI-TOF-MS and linkage analysis on GC/MS is capable of providing these data.

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363: Comparative biochemical analysis of planarian N-glycans

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Whereas structures of N-glycans are generally diverse, variation of the diversity through the phylogeny is still unknown. In particular, structural information from invertebrate glycans is very limited. The nematode, *Caenorhabditis elegans* has unique and various N-glycans, though the worm has a very simple body construction. The major N-glycans in the cephalopods, squid and octopus are similar to the complex-type glycans of vertebrates. In contrast, gastropods, which belong to the mollusk family as do the cephalopods, have quite different N-glycans modified with methyl residues. This knowledge suggests that phylogenic closeness and N-glycan similarity are not correlated in a simple way. In this study, to investigate the relationship between phylogeny and glycan structures, we analyzed the structure of planarian N-glycans. Planarian, which belongs to the flatworm family, is a lower metazoan. The N-glycans were prepared from whole worm by hydrazinolysis, followed by tagging with the fluorophore, 2-aminopyridine at their reducing end. The labeled N-glycans were purified away from contaminants, and separated by three different steps of high performance liquid chromatography. By comparison with standard pyridylaminated N-glycans, it was demonstrated that they included high mannose-type and pauci-mannose-type glycans. However, it was estimated that many of the major N-glycans from planarians have novel structures, since their elution positions did not match those of the standard glycans. The results of mass spectrometry and sugar component analyses indicated that these glycans included methyl mannoses and the most probable linkage style of them was 3-O-methylation. Furthermore, the methyl residues on the most abundant glycan could be attached to the non-reducing end mannose, since the glycans were resistant to α-mannosidase digestion. Those results indicate that the planarian have the methylated high-mannose type glycan as the most abundant structure.

364: Presence of galactosylated core fucose on N-glycans in the planaria *Dugesia japonica*

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Planarial species are of especial interest to biologists due to the phenomenon of pluripotency and, in comparison to other developmental processes, it can be hypothesised that glycan-lectin interactions may play a role. In order to examine the N-glycans of one of these organisms, *Dugesia japonica*, peptide:N-glycosidase A was employed and the released glycans were subject to pyridylamination, HPLC and mass spectrometric analysis. A range of oligomannosidic glycans was observed with a trimethylated $Man_5GlcNAc_2$ structure being the dominant species. Three glycans were also observed to contain deoxyhexose; in particular, a glycan with the composition $Hex_4HexNAc_2Fuc_1Me_2$ was revealed by exoglycosidase digestion, in combination with MS/MS, to contain a galactosylated core α1,6-fucose residue, whereas this core modification was found to be capped with a methylhexose residue in the case of a $Hex_5HexNAc_2Fuc_1Me_3$ structure. This is the first report of these types of structures in a platyhelminth and indicates that the 'GalFuc' modification of N-glycans is not just restricted to molluscs and nematodes. (See: Paschinger *et al.*, 2011, Journal of Mass Spectrometry, in press.)

365: Analysis of N-glycans of the Eastern Oyster *(Crassostrea virginica)*

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The Eastern Oyster (*Crassostrea virginica*) is a resident mollusc off the shores of the eastern coast of the United States and was formerly of great commercial value – however, a number of factors such as over-harvesting, pollution and disease have devastated stocks. One of these diseases is Dermo disease caused by the protozoan *Perkinsus marinus*, which degrades a major plasma defence protein in the oyster (so-called dominin) and is taken up by the major immune cells (haemocytes) in the haemolymph via a galectin-dependent system. In order to examine the glycomic potential of the host organism, the N-glycans were released from glycopeptides prepared from both the plasma and haemocytes. Normal-phase HPLC analysis in combination with MALDI-TOF MS suggested the presence of two major subsets of glycans with those of earlier retention time showing mass differences, as compared to those of later retention time, of *m/z* 80 or 102. Selected fractions were subject to exoglycosidase digestions and MS/MS – the data were compatible with 'blocking' of antennal galactose residues by sulphation of the earlier-eluting glycans. Furthermore, some of the galactose residues were modified by methylated and non-methylated α-N-acetylgalactosamine. Some of core and peripheral N-acetylglucosamine residues were fucosylated; in the haemocytes, there is also evidence of core difucosylation of a subset of N-glycans as well as some oligomannosidic forms. In summary, the Eastern Oyster expresses a range of apparently novel N-glycans, with sulphate, GalNAc and fucose residues, on its plasma and haemocyte proteins.

366: Comparative N-glycomics of Dictyostelium strains and species

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Over the last three decades the elucidation of the N-glycans of the cellular slime mould *Dictyostelium discoideum* was a challenge for many research groups. The changes during its life cycle, from a unicellular to a multicellular organism, were revealed to be also interesting concerning the N-glycome.The N-Glycans obtained from four different stages of development, differed in size and presence of intersected and bisected N-acetylglucosamine residues. The presence of an α 1,3 linked fucose was constant through all stages of development. The axenic wild type Ax3 N-glycans were analysed after enzymatic release by HPLC and MALDI MS and MS/MS. Charged N-glycans containing methylphosphate and sulphate could be detected in the axenic as well as in the mutant strains. For a better understanding of the N-glycome, we analysed several glycosylation mutant strains. One of them (HL241) has a defect on the gene (alg9) coding for an enzyme active during the N-glycan precursor ($Glc_3Man_9GlcNAc_2$) biosynthesis. This strain accumulates N-glycans of a reduced size, specifically $Hex_6GlcNAc_2$. The natural isolate NC4, was also analysed, as well as two other species *D. purpureum* and *D. giganteum*. Comparing this species at a late developmental stage (fruiting bodies), we found core fucosylated and rather smaller N-glycans than at the cellular stage.The N-glycans of the slime mould *D. discoideum* are as fascinating as the organism itself. The comparison of the N-glycosylation of the Dictyostelium species might help us to know more about the diversity of slime moulds. On the other hand the mutant strains will reveal more about single enzymes, and how their malfunction is affecting N-glycosylation of the organism.

367: Five species of social amoebae display qualitatively unique N-glycomes that may support developmental autonomy in soil environments

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Cellular slime molds (CSMs) live as free amoebae in overlapping subterranean environments near the soil surface. Despite similar life-styles, individual species form independent starvation-induced fruiting bodies whose spores can renew the life cycle. We have hypothesized that a glycan shroud associated with the cell surface glycocalyx contributes to intercellular recognition and avoidance. N-glycans from five CSM species whose genomes have been sequenced were generated by pepsin digestion followed by PNGase A, fractionated into neutral and acidic pools by anion exchange, and profiled by MALDI-TOF-MS. As reported recently by Iain Wilson's group and ourselves, growing amoebae of *Dictyostelium discoideum* (Dd) express modestly trimmed high mannose N-glycans that can be peripherally decorated with 1-2 GlcNAc and 1-2 fucose residues, as well as 1-2 methylphosphates and/or sulphates. Comparative analyses of *D. purpureum*, *D. fasciculatum*, *Polysphondylium pallidum*, and *Actyostelium subglobosum* reveal that each CSM displays a characteristic signature of high-mannose species with quantitative and qualitative differences relative to D. discoideum. Differences include reduction or absence of peripheral GlcNAc and core fucose, increased fucosylation, increased Man-trimming, novel

mannose methylation, and increased or decreased anionic modifications. Starvation-induced developmental changes include further mannose-trimming, increased fucosylation, and decreased abundance of methylphosphates and sulfates. Glycan structure models derived from the MALDI-TOF-MS analyses were tested using linkage specific antibodies, exoglycosidase digestions, MS/MS, and chromatography studies. Bioinformatics analyses point to the identities of genes responsible for mediating peripheral GlcNAc and core-fucose additions, which will enable future reverse genetic studies to eliminate N-glycomic differences to test their functions in interspecific relations.

368: Chemical characterization of the oligosaccharides in Bactrian camel *(Camelus bactrianus)* milk and colostrum

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Bacterian camel milk and colostrum are commonly used as foods in Mongolia, whose people believe that these products promote human health. It has been hypothesized that milk oligosaccharides are biologically significant components of human milk, acting as receptor analogs that inhibit the attachment of pathogenic microorganisms to the colonic mucosa, and as prebiotics, which stimulate the growth of bifidobacteria within the infant colon. To evaluate their biological significance, we studied the oligosaccharides present in samples of Bacterian camel milk and colostrum. Using ^{1}H-NMR, we identified and characterized the following oligosaccharides of camel colostrum: Gal(β1-4[Fuc(α1-3)]Glc (3-FL), Gal(β1-3)Gal(β1-4)Glc (3'-GL), Gal(β1-6)Gal(β1-4)Glc (6'-GL), Neu5Ac(α2-3)Gal(β1-4) Glc (3'-SL), Neu5Ac(α2-6)Gal(β1-4)Glc (6'-SL), Neu5Ac(α2-3)Gal(β1-3)Gal(β1-4)Glc (sialyl-3'-GL), Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc (LST c), Neu5Ac(α2-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)] Gal(β1-4)Glc (sialyllacto-N-novopentaose a), Gal(β1-3) [Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (sialyllacto-N-novopentaose b) and Neu5Ac(α2-6) Gal(β1-4)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β1-6)] Gal(β1-4)Glc (monosialyl LNnH). The oligosaccharides in mature camel milk were characterized as 3'-GL, Gal(β1-3) [Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novopentaose 1) and 3'-SL. Reference: K. Fukuda et al., J. Dairy Sci. 93, 5572-5587, 2010.

369: Heparan and Chondroitin Sulfates from Earthworms *(Eisenia andrei)*

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Acidic polysaccharides, especially glycosaminoglycans, of earthworms have not been reported previously in any detail. We extracted glycosaminoglycan-rich fraction from whole tissue of the earthworm (*Eisenia andrei*). Two fractions (1 M and 2 M NaCl fractions) eluted from an anion-exchange chromatography showed the presence of acidic polysaccharides on agarose gel electrophoresis. Monosaccharide compositional analysis showed that galactose and glucose were most abundant in both fractions. Liquid chromatography–electrospray ionization mass spectrometry (LC–ESI–MS) was performed to obtain disaccharide compositional analysis in 2 M NaCl fraction after chondroitinase digestion. The result showed that the chondroitin sulfate contained a 4-O-sulfo (76%), 2,4-di-O-sulfo (15%), 6-O-sulfo (6%), and unsulfated (4%) uronic acid linked N-acetylgalactosamine. LC-ESI-MS analysis showed the presence of N-sulfo (69%), N-sulfo-6-O-sulfo (25%) and 2-O-sulfo-N-sulfo-6-O-sulfo (5%) uronic acid linked N-acetylglucosamine after heparin lyase I/II/III digestion. Average molecular weight of 2 M NaCl fraction was 5,800 Da measured by high-performance size exclusion chromatography.

370: Chondroitin Sulfates from Marine Organisms

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We investigated the by-products of marine organisms (giant squid, salmon, skate, flatfish, and yellow goosefish) to search for chondroitin sulfates (CS) as new marine sources. Depolymerization with chondroitinase showed that purified CS did not contain other glycosaminoglycans on agarose gel electrophoresis. The structure and purity of CS were confirmed by ^{1}H-NMR. The average molecular weight ranging from 22 to 116 kDa was determined by high-performance size exclusion chromatography. Strong anion exchange-high performance liquid chromatography (SAX-HPLC) was performed to obtain disaccharide composition and purity after chondroitinase digestion which demonstrates the purity (81.7±1.3 to 114.2 ± 2.5%) and the yield (1.3 to 21.6%) were varied on depending on sources. From all data, CS from giant squid cartilage, salmon cartilage, skate cartilage, and yellow goosefish bone could be promising marine sources to substitute shark cartilage CS in commercial neutraceuticals.

371: Glycosaminoglycan-binding activity of ZG16p and ZG16B/PAUF

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ZG16p was first identified as a soluble 16 kDa pancreatic protein having remarkable sequence homology to β-prism fold plant lectins such as Man/Glc-specific BanLec from banana and Gal/GalNAc-specific jacalin from jack fruit. ZG16p is thought to be involved in sorting of digestive enzymes to zymogen granules in the exocrine pancreas by interacting with glycosaminoglycans. Recently, we determined the crystal structures of human ZG16p and ZG16B/PAUF, a paralog of ZG16p, and revealed that the three-dimensional structure of these proteins is a jacalin-related β-prism fold (Biochem Biophys Res Commun 404(2011)201-205). ZG16B/PAUF is highly expressed in human pancreatic cancer and plays a role in cancer metastasis, although its glycosaminoglycan-binding activity has not been examined.

In this study, we expressed recombinant human ZG16p and ZG16B/PAUF in *E. coli*, and compared their glycosaminoglycan-binding activities by solid-phase binding assays using bovine serum albumin-conjugated glycosaminoglycans. ZG16p and ZG16B/PAUF bound to heparin in a dose-dependent manner and to a lesser extent to heparan sulfate, although the relative binding activities of ZG16B/PAUF to heparin and heparan sulfate were lower than those of ZG16p. Among chondroitin sulfates (CS)-A, CS-B, and CS-C, ZG16p and ZG16B/PAUF preferentially bound to CS-B. In plant β-prism fold lectins, GG and GXXXD loops are conserved as ligand-binding motifs and cooperatively form the carbohydrate-binding site. ZG16p, but not ZG16B/PAUF, has one set of GG and GXXXD loops. Site-directed mutational analysis of ZG16p showed that basic amino acid residues localized near the GG and GXXXD loops contribute to the interaction of ZG16p with glycosaminoglycans. To analyze the expression of ZG16p and ZG16B/PAUF, we performed PCR of cDNA samples from various human tissues and found that the pancreas and colon are major expression sites for both ZG16p and ZG16B/PAUF, while only ZG16B/PAUF is expressed in the prostate and lung.

372: Three Mechanisms for *Agaricus bisporus* Agglutinin– Glycan Recognitions (ABA)

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For the GalNAcα1→ specific *Agaricus bisporus* agglutinin (ABA) from an edible mushroom, the mechanism of polyvalent Galβ1→3/4GlcNAcβ1→ complex in ABA–carbohydrate recognition has not been well defined since Gal and GlcNAc were weak ligands. By enzyme-linked lectinosorbent and inhibition assays, it is shown the polyvalent Galβ1→3/4GlcNAcβ1→ in natural glycans also play vital roles in binding and proposed that four different intensities of glycotopes (Galβ1-3GalNAcα1-, GalNAcα1-Ser/Thr and Galβ1-3/4GlcNAcβ1-) construct three recognition systems at the same domain. This peculiar concept provides the most comprehensive mechanism for the attachment of ABA to target glycans and malignant cells at the molecular level.

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373: Galectin-8-N-domain recognition mechanism for sialylated and sulfated glycans

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Galectin-8 has much higher affinity for 3′-O-sulphated or 3′-O-sialylated glycoconjugates and a Lewis X-containing glycan than for oligosaccharides terminating in Galβ1-3/4GlcNAc, and this specificity is mainly attributed to the N-terminal carbohydrate recognition domain (N-domain, CRD) (Ideo H, Seko A, Ishizuka I, Yamashita K. (2003) Glycobiology 13, 713–723). In this study, we elucidated the crystal structures of human galectin-8-N-domain (-8N) in the absence or presence of 4 ligands. The apo molecule forms a dimer, which is different from the canonical 2-fold symmetric dimer observed for galectin-1 and -2. In a galectin-8N–lactose complex, the lactose-recognising amino acids are highly conserved among the galectins. However, Arg45, Gln47, Arg59, and the long loop region between the S3 and S4 β-strands are unique to galectin-8N. These amino acids directly or indirectly interact with the sulfate or sialic acid moieties of 3′-sialyl- and 3′-sulpho-lactose complexed with galectin-8N. Furthermore, in the LNF-III–galectin-8N complex, van der Waals interactions occur between the α1-3 branched fucose and galactose and between galactose and Tyr141, and these interactions increase the affinity toward galectin-8N. Based on the

findings of these X-ray crystallographic analyses, a mutagenesis study using surface plasmon resonance showed that Arg45, Gln47, and Arg59 of galectin-8N are indispensable and coordinately contribute to the strong binding of galectins-8N to sialylated and sulphated oligosaccharides. Arg59 is the most critical amino acid for binding in the S3~S4 loop region.

374: Systematic analyses of free ceramide species and ceramide species comprising neutral glycosphingolipids by MALDI-TOFMS/MS2 with high-energy CID

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Free ceramides and glycosphingolipids (GSLs) are important components of the membrane microdomain and play significant roles in cell survival. Namely, free ceramides, sphingomyelins and their metabolites such as sphingosine and sphingosine-1-phosphate are recognized as signaling molecules involved in cell differentiation, proliferation and apoptosis. GSLs, which are also involved in cell signaling processes, mediate cell–cell adhesion and pathogen entry. Recent studies have revealed that both fatty acids and long-chain bases (LCBs) are more diverse than expected, in terms of i) alkyl chain length, ii) hydroxylation and iii) the presence or absence of double bonds. These factors are considered to affect the geometry of sphingolipids in membrane microdomains of cells. More importantly, evidence is rapidly accumulating to suggest that specific molecular species in a single class of certain lipid exhibit particular functions. Electrospray ionization mass spectrometry and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) have been well utilized to characterize sphingolipids with high throughput, but reports to date have not fully characterized various types of ceramide species such as hydroxyl fatty acids and/or trihydroxy-LCBs of both free ceramides and the constituent ceramides in neutral GSLs. We performed a systematic analysis of both ceramide species, including LCBs with non-octadeca lengths using MALDI-TOFMS with high-energy collision-induced dissociation (CID) at 20 keV. Using both protonated and sodiated ions, this technique enabled us to propose general rules to discriminate between isomeric and isobaric ceramide species, unrelated to the presence or absence of sugar chains. In addition, this CID method can present some linkage information about oligosaccharides. Using this method, we demonstrated distinct differences among ceramide species, including free ceramides, sphingomyelins, and neutral GSLs of GlcCers, GalCers, LacCers, Gb3Cers and Forssman glycolipids in the equine kidneys.

375: Fucosylated monosialogangliosides with polyglycosylceramide cores are preferential receptors of human H3N2 influenza A viruses Victoria/3/75 and Hiroshima/52/2005

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Influenza represents a serious human burden causing annual half million deaths worldwide. Among influenza A, B, and C types, subtype A is the most virulent and is associated with seasonal epidemics. Influenza A viruses are classified by the two surface proteins hemagglutinin (HA) and neuraminidase (NA), encompassing 16 HA (H1-H16) and 9 NA (N1-N9) subtypes. H3N2 virus strain A/Victoria/3/75 caused an estimated 11,000 deaths during the 1976 influenza season and the closely related H3N2 strain A/Hiroshima/52/2005 has been used since his first isolation as a H3N2 vaccine strain. Influenza virus infection is initiated via viral HA that binds to terminally α2-3- and/or α2-6-sialylated glycoconjugates such as glycosphingolipids (GSLs). In this study, we used sialylated GSLs (gangliosides) from human granulocytes for probing their binding potential to A/Victoria/3/75 and A/Hiroshima/52/2005 influenza strains. Techniques employed for receptor identification and their structural characterization were the thin-layer chromatography (TLC) overlay assay and nanoelectrospray ionization mass spectrometry (nanoESI MS), respectively. Surprisingly, TLC overlay assay binding studies with both H3N2 viruses gave negative results for gangliosides with nLc4Cer and nLc6Cer cores carrying α2-3- or α2-6-linked N-acetylneuraminic acid. Interestingly, highly polar gangliosides with low TLC mobility composed of neolacto-cores with 8 or more monosaccharides exhibited strong virus adhesion. Prior sialidase treatment completely abolished virus binding indicating strict requirement of sialic acid for virus binding. Auxiliary TLC overlay assays using antibodies specific for sialyl-Lewis^x (sLe^x) epitopes showed identical binding patterns compared to those performed with the two viruses. The detailed structural analysis of virus binding gangliosides revealed sLe^x-gangliosides with nLc8-, nLc10- and nLc12Cer-cores carrying one to three fucose residues, respectively. This is the first report that describes sLe^x-gangliosides from human cells acting as receptors for H3N2 viruses. Thus, our results suggest involvement of fucosylated neolacto-series monosialogangliosides in host-pathogen interaction of influenza viruses and in particular shed new light on the diversity of potential ganglioside receptors of H3N2 influenza viruses.

376: Sequence determination by negative-ion electrospray tandem mass spectrometry of monosialylated ganglio-oligosaccharides

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Gangliosides, as plasma-membrane bound sialylated glycolipids, serve as ligands for endogenous proteins (e.g. siglecs) and adhesion proteins of pathogens (viruses, bacteria and parasitic agents) and toxins of bacteria. To investigate ganglio-oligosaccharide sequences as ligands, we have underway a programme to develop a neoglycolipid (NGL)-based (1,2) 'gangliome' microarray using oligosaccharide populations released from gangliosides. Assignments of carbohydrate ligands using glycolipids can be complicated by the heterogeneities of the lipid moieties (3). Due to the presence of lipids, particularly in their multiple forms, HPLC separation and purification, and sequence determination of glycolipids can be difficult. We developed a strategy for high sensitivity (1-5 pmol) sequencing of mono-sialylated ganglio-oligosaccharides based on negative-ion electrospray mass spectrometry with collision-induced dissociation (ES-CID-MS/MS) (4). In the product-ion spectra of singly charged precursor $[M-H]^-$, several unique fragment ions (D_1, D_2, T_1 and T_2) derived from double- and triple-cleavages were identified and these are specific for mono-sialylated ganglio-oligosaccharides. Different fragmentation patterns were observed in the product-ion spectra of doubly charged precursors $[M-2H]^{2-}$, which gave clear sequence information. Combined use of the ES-CID-MS/MS of singly and doubly charged molecular ions are important for identification and assignment of the sequences of glanglio-oligosaccharides. Using this ES-CID-MS/MS strategy we have identified several minor components within the mono-sialylated ganglio-oligosaccharide populations released by ozonolysis from bovine brain gangliosides and group-fractionated by anion-exchange chromatography. This work was supported by a UK MRC grant (G0600512) and a U.K. BBSRC grant (BB/E02520X/1).

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377: First Principles Calculations of Novel Anti-parainfluenza Drugs using Fragment Molecular Orbital Method

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Human parainfluenza virus (hPIV) is a member of Paramyxoviridae family, causing the common cold with fever, croup, bronchitis, and pneumonia in infants and children. Four different types of hPIVs have been identified, all of which cause a spectrum of illnesses of the upper and lower respiratiry tracts of children. However, no vaccines or specific therapies for infections caused by these viruses are currently available. Hemagglutinin-neuraminidase (HN) glycoprotein is a good target for chemical treatment of hPIV infectious disease. We are required to develop a curative medicine for hPIV infectious disease in modern aging society, because there is danger of infecting the senior citizen and socially vulnerable. To develop new sialidase inhibitors against hPIV, we proposed novel drug discovery procedure. This procedure is based on the ab inito fragment molecular orbital (FMO) method. We applied the procedure to analyze the interaction of sialic acid derivatives with the HN glycoprotein of hPIV. We performed in silico analysis at ab initio total electronic MO calculation. This MO calculation can correctly evaluate both hydrophilic and hydrophobic interactions between the derivatives and amino acid residues in target protein, and it does not include empirical parameters. Recently, one of collaborators, Ikeda reported syntheses of several sialic acid derivatives, which had effective sialidase inhibitory activities against hPIV. The multifunctional role of HN glycoprotein in the viral life cycle makes it an attractive target for the development of chemotherapeutics to treat hPIV infection. We have showed the critical interactions between the derivatives and amino acid residues in hPIV HN glycoprotein. We revealed the important factors of the potential inhibitory activity. We also carried out de novo virtual screening of sialidase inhibitors and several compounds by using open database ZINC. We could theoretically predict several candidate compounds which have highly inhibitory activity against hPIV. The novel compounds expected as curative medicine for hPIV infectious disease are theoretically predicted.

378: First principles calculations of influenza neuraminidase mutants and anti-influenza drugs using ab initio fragment molecular orbital method

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An anti-influenza drug, Oseltamivir (Tamiflu®) is currently and widely used to treat infected patients. In recent years, drug-resistant mutants of influenza neuraminidase (NA) have been reported, and the X-ray crystal structures of the complexes of the H274Y/N294S NA mutants with Oseltamivir have been determined. However, the molecular mechanism of the Oseltamivir-resistance of the mutants has not yet been elucidated. We performed first principles calculations about the complexes of the mutants with Oseltamivir based on ab initio Fragment Molecular Orbital method to explore why the mutants are resistant to Oseltamivir. We quantitatively analyzed interaction energies between Oseltamivir and amino-acid residues composed of the mutants. We evaluated the interaction energies between Oseltamivir and amino-acid residues composed of the wild type (WT) and H274Y/N294S mutants. When the WT mutated, the interaction energies of Oseltamivir with the mutated amino acid residues have not changed. In the case of the H274Y mutant, the change of the mutated residue-adjacent residues interaction is not enough small, and the change of Oseltamivir-adjacent residues interaction is large. In the case of N294S mutant, the change of the mutated residue-adjacent residues interaction is not small, but the change of Oseltamivir-adjacent residues interaction is small. We found that Oseltamivir-resistance is caused by changing of key interaction energies between the mutated amino acid residue and its adjacent residues, but not Oseltamivir directly. We also analyze the molecular origin of permissive secondary mutations. The evolution of Oseltamivir-resistance was enabled by "permissive mutations R194G, R222Q and V234M". These mutations allowed the virus to resistant after H274Y occurred. To understanding this process may provide a basis for predicting the evolution of Oseltamivir-resistance in other influenza strains. When the WT mutated R194G, the interaction energies between Oseltamivir and amino acid residues have not changed. On the other hand, when a secondary mutation occurred, the energies have significantly changed.

379: Theoretical study on catalytic reactivity of the M344H mutant of inverting β-1,4-galactosyltransferase-1.

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The inverting β-1,4-galactosyltransferase-1 (β4Gal-T1) requires for catalytic activity a Mn^{2+} ion cofactor. In the active site of β4Gal-T1, Mn^{2+} is bound in an octahedral coordination with the Asp254 residue of the DXD motif, the Met344 and His347 residues, one molecule of water and two diphosphate oxygens of the UDP galactose substrate. In the contrast, Mg^{2+} does not activate the wild-type enzyme. In the mutant M344H 25.0 % of the catalytic activity was observed in the presence of Mg^{2+}, while in the presence of Mn^{2+} only 1.5 % of the wild type enzyme activity was found [1]. To understand catalytic effects of the M344H mutation and the substitution of Mn^{2+} by Mg^{2+} on chemical reactivity of β4Gal-T1, density functional theory (DFT) calculations at the quantum mechanics (QM) and hybrid QM/MM levels were performed modeling interactions in a complex enzyme-ion cofactor and modeling the enzymatic reaction step of a galactose residue transfer from the UDP-Gal donor to the N-acetylglucosamine acceptor. Financial support for this research was granted by the Scientific Grant Agency of the Ministry of Education of Slovak Republic and Slovak Academy of Sciences (projects VEGA-02/0176/09 and VEGA-02/0101/11).

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380: Automated implementation of site, epitope and conformational mapping of carbohydrate-protein complexes: binding mode determination using the Carbmap procedure

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Although structural knowledge of carbohydrate-protein complexes is useful for a wide variety of design programs (and used successfully in the development of Relenza™), obtaining this knowledge can be experimentally challenging due to the flexibility of carbohydrates. To address this, we have developed Carbmap, a fully automated implementation of our previous work in delineating the nature of these interactions. The Carbmap procedure requires as input an ensemble of carbohydrate poses obtained from molecular docking. Although poses from any molecular docking program may be used, Glide and GOLD are the preferred options as these have been demonstrated to perform the best for carbohydrate-protein docking. The ensemble is analysed to determine the most likely protein residues involved in ligand recognition, and the most likely ligand atoms recognised by the protein. The component disaccharide linkages of the carbohydrate ligands are analysed to determine likely conformational states. The set of interactions taking place in each pose is compared to every other pose in the ensemble to identify poses placed in similar spatial locations. The combination

of these filters is used to select likely carbohydrate binding modes. The protocol has been evaluated for carbohydrate-antibody complexes (including complexes with sugar acids), and is currently being investigated for application to peptide-antibody complexes and carbohydrate-lectin complexes.

381: Characterisation of arabinogalactan-proteins isolated from suspension cultures of *Pelargonium sidoides* DC

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Pelargonium sidoides is a traditional medicinal plant from South Africa. An aqueous-ethanolic formulation of the roots is approved for the treatment of acute bronchitis. The main effects could be related to antibacterial activities and the stimulation of the non-specific immune system by the main components of *P. sidoides*: coumarins, phenols and tannins [1]. Due to wild harvesting, *P. sidoides* is an endangered species. Therefore the propagation of the plant material by cell cultures and the extraction of ingredients are interesting tasks. In suspension cultures of *Echinacea purpurea* a secretion of arabinogalactan-proteins (AGPs) with weak immunomodulatory activities has been shown [2]. From suspension cultures of *P. sidoides* high amounts of pure AGPs could be isolated by precipitation with β-glucosyl Yariv reagent. To study potential therapeutic benefits the characterisation of these AGPs is necessary. Quantification of neutral sugars by acetylation pointed out arabinose (Ara) and galactose (Gal) as dominating monosaccharide residues in a ratio of 1 : 2. Colourimetric determination of uronic acids revealed an amount of 6-8%. Linkage type analysis in combination with the reduction of the uronic acids showed that the main components are 1,3,6-Gal*p*, 1,3-Gal*p* and 1-Ara*f* as well as minor amounts of 1,6-Gal*p*, 1-GlcA*p*, 1,4-Gal*p*, 1-Gal*p*, 1,5-Ara*f* and 1,2-Ara*f*. Molecular weight of AGPs has been determined by size exclusion chromatography with laser light scattering detection and found to range between 80 and 85 kDa. The characterisation of the AGP-protein moiety pointed out an untypical low protein content for AGPs with 1%. According to the amino acid analysis the protein moiety consists of high amounts of Hyp (42.8-51.1%) as well as Pro, Gly, Glx, Asx, Ser, Ala, Leu and Thr.

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382: Heterogeneity of exopolysaccharides from cyanobacteria of the genus *Synechocystis*

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Cyanobacteria can synthesize exopolysaccharides (EPS) in various amounts and of high variability with 2 - 9 different monosaccharides [1]. Up till now only two amino sugars are known to occur: N-acetyl-glucosamine and N-acetyl-galactosamine [2]. Further amino sugars like mannosamine and muramic acid are components of peptidoglycans from cell wall layers [3]. For the first time we identified fucosamine (2-amido-2,6-dideoxy-D-galactose) as a component of the EPS of a cyanobacterium, namely *Synechocystis aquatilis* SAG 90.79 (*S. aquatilis*). The monosaccharide composition of the EPS of *S. aquatilis* is very different from those of other known cyanobacteria [2] and even of that of the nearly related species *Synechocystis pevalekii* SAG 91.79 (*S. pevalekii*). The acidic EPS (~20% sulfate groups) from *S. aquatilis* mainly consists of only four sugars: fucose (42%), arabinose (34%), fucosamine (17%) and glucose (1%). In contrast the EPS of *S. pevalekii* with more than seven sugars rather is comparable to other cyanobacterial EPS [2] and contains mannose (25%), glucose (19%), galactose (12%), fucose (10%), rhamnose (8%), xylose (8%), glucosamine (5%) and arabinose (3%). Beside polysaccharides also proteins are mentioned to occure in ethanol-precipitated fractions of purified cultivation medium [4]. We detected higher amounts of proteins in the isolated EPS fraction of S. pevalekii. In contrast the EPS fraction of S. aquatilis is free of protein moieties. Our results show again the high heterogeneity of cyanobacterial exometabolites even within one species. The occurrence of fucosamine in the EPS of a cyanobacterium once more confirms the integration of cyanobacteria into the group of gram-negative bacteria.

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383: Size-exclusion chromatographic study of the molecular-weight distribution of exopolysaccharides dextran, pullulan, and their metal(II)-complexes

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Biometal complexes with exopolysaccharides like dextran and pullulan, are important in both veterinary and human medicine for the treatment of different anaemia types [1]. The optimization and the entire process of the low-molecular weight dextran and pullulan arrangement, as well as the reaction mechanism of polysaccharides with Cu(II) and Co(II) ions have been described in detail by Mitic et al. [1,2]. Development and validation of a Size-Exclusion Chromatographic (SEC) method for dextran, pullulan, and their Cu(II) and Co(II) [M(II)] complexes in solution, was described in this work. The SEC method was adopted from standards on the Dextran 5, 10, 40, and

Dextran 70 materials. The SEC method was performed under isocratic conditions and optimized with a mobile phase flow rate of 1 mL/min, and column temperature of 40 °C, to sharpen dextran and glucose peaks. SEC is useful method to study the molecular weight distribution, polydispersity, polymerization degree of polysaccharides, and their biometal complexes. The SEC method was capable of detecting changes in molecular weight distribution caused by complexation under extreme alkaline conditions and heat, thereby confirming the stability indicating nature of the method. Molecular weight distribution of polysaccharides were unchanged when the dextran or pullulan containing test solutions were subjected to forced degradation using heat, light (daylight and UV light), extreme acidic, alkaline or oxidative conditions. The concentration of polysaccharides, metal content, and solution pH did not affect the measured molecular weight distribution of dextran and pullulan in their biometal complexes.

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384: Identification of glycosylated sites of polysaccharide-protein conjugate vaccines

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The mapping of glycosylation sites represents a fundamental key aspect to develop synthetic procedures for preparation of well-defined conjugates and to understand the role of glycopeptides in the vaccine immunogenicity [1, 2]. Since the analytical evaluation and structural characterization of these vaccines are highly dependent from the physicochemical methods [3, 4], the mass spectroscopy undoubtedly represents a very powerful tool for this kind of characterization. A robust analytical procedure to identify which amino acids are glycosylated is consequently a key methodology. The identification of glycosylated amino acid should be useful for further optimization of conjugation parameters, such as the coupling chemistry to be used, the linkers, etc. This methodology is based on the enzymatic digestion of the protein component of glycoconjugate, a mixture of peptides and glycopeptides is obtained, a subsequent cleavage of the saccharide chains which generates glycopeptides with a single saccharide adduct, and their final assignment by LC-ESI-MS. Development of the analytical method and application on glycoconjugates vaccines such as antigen against *Neisseria meningitidis*, group B *Streptococcus*, *Candida albicans*, are reported.

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385: Crystallization Experiments of Selected Components from the S-Layer Glycosylation Pathway of *Paenibacillus alvei* CCM 2051T

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Paenibacillus alvei CCM 2051T is a Gram-positive, mesophilic bacterium that is completely covered with a glycosylated surface (S) layer protein (SGP). The O-glycosidically linked S-layer glycans are polymers of [-3)-β-D-Gal-(1[α-D-Glc-(1-6)]-4)-β-D-ManNAc-(1-] repeating units that are linked via the adaptor GroA-2-OPO2-4-β-D-ManNAc-(1-4)]-3)-[α-L-Rha-(1-3)]$_n$=3 β-D-Gal-(1- to specific tyrosine residues of the S-layer protein. The S-layer protein itself is non-covalently linked by a secondary cell wall polymer (SCWP) with the structure 3)-[4,6-O-(1-carboxyethyldiene)]-β-D-ManNAc-(1-4)-β-D-GlcNAc-(1- to carbon 6 of N-acetylmuramic acid residues of the peptidoglycan. The genes involved in the glycosylation process are clustered in distinct, closely spaced slg (S-layer glycosylation) gene loci comprising approximately 24 kb of known sequence. Usually S-layer proteins are insoluble in aqueous environment and have the inherent ability to rapidly crystallize in large two-dimensional lattices, either on intact cells or in vitro. Therefore, 3D crystallization experiments of the complete S-layer proteins have been unsuccessful so far. Recently it has been demonstrated that SCWPs are able to render isolated S-layer proteins soluble in aqueous systems. Since the SGP / SCWP complex of *P. alvei* CCM 2051T obtained after purification of the S-layer protein from native host cells has shown to be water soluble, X-ray crystallography will be applied for characterization of the type of interaction between SGP and SCWP and, thus, the attachment of the SGP to the cell wall. The work is supported by the Austrian Science Fund, projects P21954-B20 (to CS), P22791-B11 (to PM), and and BioToP W1244 to (PM & CS).

386: Crystal structure of GalNAcβ1–4GlcNAc-specific lectin from basidiomycete *Clitocybe nebularis* that inhibits Jurkat human leukemic T cells

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Basidiomycete fungi constitute a rich source of lectins exhibiting various carbohydrate-binding properties and structures. We have isolated a novel ricin B-like lectin from the basidiomycete mushroom clouded agaric (*Clitocybe nebularis*) using affinity chromatography on immobilized lactose. The 15.9-kDa lectin was designated *Clitocybe nebularis* lectin (CNL). The complete gene (EU682006) and cDNA (FJ477895) nucleotide sequences were obtained, and active recombinant CNL was expressed in *Escherichia coli*. Glycan microarray analysis revealed that the lectin is specific for N,N'-diacetyllactosediamine (GalNAcβ1–4GlcNAc) and human blood group A determinant-containing carbohydrates (GalNAcα1–3(Fucα1–2)Galβ), which was confirmed by haemagglutination assay. Crystallographic analysis of CNL in a complex with lactose and N,N'-diacetyllactosediamine revealed a β-trefoil fold composed of three homologous subdomains (α, β and γ). The CNL structure is homodimeric, with one sugar-binding site per domain in the α-subdomain. To study the mode of action of CNL, mutants were constructed on the basis of the crystal structure. A mutant that does not bind carbohydrates (A-CNL) and mutants that do not form dimeric structures (Mono-CNL) were expressed in bacteria *E. coli*. We have shown that CNL inhibits human leukemic T lymphocytes (Jurkat cell line) in a dose-dependent manner, the number of viable leukemic cells being significantly reduced – ~60% decrease at the highest concentration (100 μg/ml) of CNL. Since CNL did not inhibit other cells tested, we conclude that the effect is not due to general cytotoxicity of the lectin but is specific to the Jurkat cell line. When CNL was preincubated with 0.1 M lactose the inhibitory effect was abolished and, moreover, A-CNL, a non-binding mutant, and Mono-CNL, non-dimerizing mutants, did not show the effect. This suggests that inhibitory effect against Jurkat cells is associated with the bivalent carbohydrate-binding property of dimeric CNL. The lectin presumably recognizes and cross-links specific glycan ligands on Jurkat cells which can lead to signal transduction and ultimately to their inhibition.

387: Synthesis of biologically active glycoconjugates of genistein

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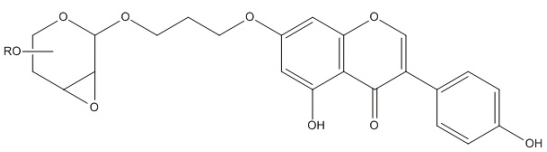
Genistein is a phytochemical with widely demonstrated beneficial effects for human health [1]. Genistein exhibits activity towards multiple molecular targets and reveals potential for affinity TuneUp by derivatization. Previous experiments, among genistein modified at C7 by different mono and di-unsaturated sugars, revealed that several compounds appeared more active than parent compound in preliminary screening for inhibition of cancer cell proliferation. The most potent glycoconjugate was Ram-3, where genistein is linked with a 2,3-unsaturated sugar moiety through an alkyl chain [2]. The role of sugar moiety as a structural element essential for antimitotic propreties of genistein derivatives is significant. In the present study we report the synthesis of new compound linking genistein with an oxirane ring in sugar moiety. Epoxidation of the sugar moiety to form a 2,3-anhydro derivative may have an important role in stereochemical molecule docking in the active site. Suitable chemical transformations, such as epoxide ring opening, give a wide range for the preparation of various interesting derivatives.



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388: Sialylglycopeptide-conjugated beads bind recombinant human influenza A virus hemagglutinin

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To develop a convenient tool for extraction and/or purification of proteins binding to human oligosaccharide chains, we studied the use of sialylglycopeptide (SGP)-conjugated beads as a ligand in a lectin-binding assay. SGP, a naturally abundant N-glycan isolated from hen's egg yolk[1], is now readily available. The Neu5Ac(α2-6) Gal residue of SGP is known to bind with type-A human influenza virus hemagglutinin.[2] Through its lysine amino groups, SGP was immobilized on beads (NHS-activated Sepharose 4 Fast Flow, GE Healthcare Bio-Sciences AB, Sweden). The physical condition of the SGP on the Sepharose beads was estimated by performing binding studies with *Sambucus sieboldiana* (SSA). The immobilized SGP was recognized by SSA lectin with specificity for the Neu5Ac(α2-6)Gal/GalNAc sequence. A constant of association (K_a) between SGP and SSA lectin of 1.1×10^6 M^{-1} was obtained by using a microtiter plate binding assay. This value is similar to the K_a value of 2.5 $\pm$ 0.4×10^7 M^{-1} obtained in a quartz-crystal microbalance assay of SSA lectin binding to the Neu5Ac(α2-6)Gal

sequence in an oligosaccharide derived from SGP.[3] The immobilized SGP also interacted with recombinant human influenza A virus hemagglutinin H1N5 protein. This interaction showed a significant difference from that in controlled experiments performed by using the same procedure without the protein.

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389: Synthesis of α-1-thioglycosides – derivatives of D-glucose, D-galactose, 2-deoxy-D-glucose and 2-deoxy-D-galactose and their use in synthesis of glycosyltransferases natural substrates analogues

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Glycosyltransferases (GTs) are enzymes responsible for biosynthesis of complex sugars and glycoconjugates. These enzymes catalyze transfer of sugar moiety from glycosyl donor to the acceptor molecule, which can be oligosaccharide, peptide, lipid or other small molecule [1]. Inhibition of glycosyltransferases have an enormous significance in controlling of synthesis of cell-surface glycoconjugates. It leads to the modulation of oligosaccharides biosynthesis and enables recognition of their biological functions. Therefore some of such inhibitors might be of therapeutic interest [2]. In the structure of GTs donor type natural substrates, three different moieties can be distinguished: carbohydrate part, diphosphate linkage and nucleoside moiety (mostly it is uridine). Our previous research on glycosyltransferases inhibitors revealed that some of synthesized sugars connected with aglycon by β-1-thioglycosidic bond exhibit biological activity against classical swine fever virus (CSFV) [3, 4]. Synthesis of GTs donor type natural substrate analogues, in which carbohydrate moiety is connected to aromatic aglycon (nitropyridine derivative) via α-1-thioglycosidic bond is very challenging and causes many difficulties. Our recent research led us to obtain glycoconjugates derivatives of D-glucose, D-galactose, 2-deoxy-D-glucose and 2-deoxy-D-galactose including 1-α-thioglycosidic part connected to selectively protected uridine through the amide bond. Research studies part-financed by the European Union within the Structural Funds in Poland (POIG.01.01.02-14-102/09).

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390: Sugar derivatives of quinoline or isoquinoline: synthesis and potential spectrum of biological activities

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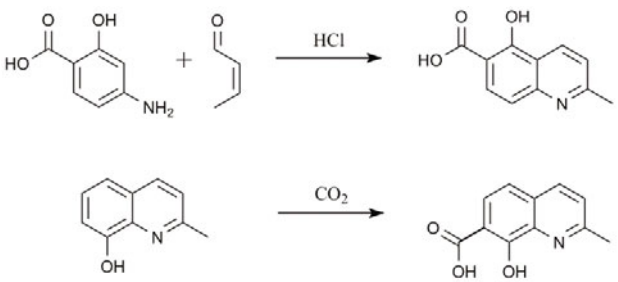
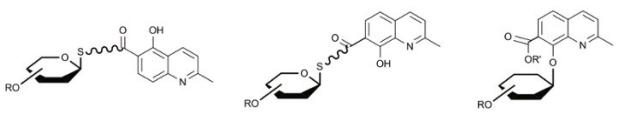
Small azanaphthalenes as quinoline or isoquinoline can be considered as privileged structures (1). They are present in many synthetic drugs and natural products with spectrum of activity covering antifungal, anticancer and antiviral effects (2-3). Nevertheless bioeffectors designed on the core of quinoline moiety still suffers from poor bioavailability/membrane transport. This prompted us to incorporate biologically relevant sugar scaffold into some quinoline related inhibitors of HIV integrase.



Sugar part could be mono- or oligosaccharide connected to quinoline derivatives by amide, ester, thioester or glycosidic bond. Choice of sugar part structure was caused by earlier performed researches (4-5).



Obtained structures and their expected and examined biological activity will be presented.

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391: Synthesis of novel sugar diamino acids

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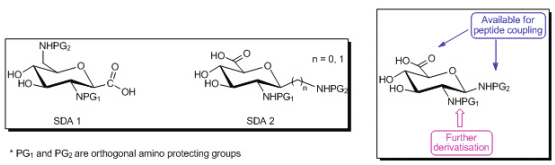
Sugar amino acids (SAAs) are found in nature as good construction elements[1] for the preparation of peptide mimetics and oligosaccharides in drug design and development. The synthesis of SAAs is readily accomplished in few steps and more than 40 SAAs have been synthesised to date.[2] Sugar amino acids with an additional amino group, the sugar diamino acid (SDAs) would represent a useful expansion to the library of SAAs available because one of the amino group and carboxylic acid is available for peptide coupling and the another amino/azide group allow to do further derivatisation via peptide or click chemistry such as labelling. However, the synthesis of SDAs is challenging and only three general type have been reported to date.[2] As part of a project involving the synthesis of novel integrin antagonists, we require a new series of SDAs to be developed. Herein, the synthesis of the novel SDAs type 1 and 2 and their applications will be presented.



Reference: (1) Gruner, S. A.; Locardi, E.; Lohof, E.; Kessler, H. Chemical Reviews 2002, 102, 491-514. (2) Risseeuw, M. D. P.; Overhand, M.; Fleet, G. W. J.; Simone, M. I. Tetrahedron: Asymmetry 2007, 18, 2001-2010.

392: Detection of N-acetylgalactosamine-containing glycosylphosphatidylinositol molecules in mammalian cells using an azide-labeled sugar analog

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Many eukaryotic cell surface proteins are post-translationally modified by a glycosylphosphatidylinositol (GPI) glycolipid that anchors the protein to the cell membrane. The biosynthesis and attachment of GPI precursors to proteins in the endoplasmic reticulum has been well defined, whereas subsequent carbohydrate side-chain modifications of protein-bound GPIs are still largely uncharacterized. N-acetylgalactosamine (GalNAc) linked to the first mannose of the core GPI glycan has been previously reported to be heterogeneously present on certain mammalian GPI-anchored proteins. However, nothing is known about the timing and mechanisms of GalNAc addition, due in part to the lack of an effective method to visualize GalNAc-containing GPIs.

Here we present a method for profiling GalNAc-containing GPIs in mammalian cells by metabolic labeling with N-azidoacetylgalactosamine (GalNAz) followed by Staudinger ligation to a biotinylated phosphine probe. This approach was validated by GalNAz labeling of endogenous and recombinant GPI-anchored proteins, followed by methodical characterization of GPIs containing the azide-activated sugar. GPI labeling with GalNAz confirms the identity of the HexNAc residue identified by mass spectrometry in GPI moieties of analyzed mammalian cells. To our knowledge, this is the first direct labeling of the GPI glycan with an azide-labeled sugar. This method permits the detection of GPIs present in the cells under normal physiological conditions, including GPI structures that are difficult to study using conventional labeling techniques. The use of this method for specific enrichment of GalNAc-containing GPIs and GPI-anchored proteins will be discussed.

393: 2-DG: Is it a 2-Deoxy-D-glucose or 2-deoxy-D-mannose? Inhibition of N-glycosylation in glioblastoma-derived cancer stem cells

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Deoxygenation of D-glucose and D-mannose at C-2 leads to structurally identical compounds known for historical reasons as 2-deoxy-D-glucose (2-DG). 2-DG is a known inhibitor of glycolysis and has been shown to interfere with the metabolism of D-glucose. Because 2-DG is also a 2-deoxy-D-mannose, we would expect that it would also interefere with mannose biosynthetic processes. Thus, we hypothesized that the 2-DG-mediated inhibition of glioma-derived stem cells (GSC11) grown under normoxic conditions results from the ability of 2-DG to affect D-glucose and D-mannose bioenergetic and biosynthetic processes. In our approach we have used mass spectrometry to analyze in detail the effects of 2-DG on the formation of N-glycans and its abillity to substitute for D-mannose in

glycosylation processes. In order to complete these studies we have synthesized 2-DG, D-glucose, and D-mannose labeled with deuterium at C-2 and C-6 and subsequently treated GSC11 cells with these monosaccharides to measure their effects on global N-glycan formation. Our results demonstrated that deuterium-labeled 2-DG was incorporated into the N-glycans, leading to the termination of oligosaccharide chain extension. Comparative glycomic analysis of control, 2-DG-treated, and D-mannose-rescued GSC11cells revealed a distinct modulation of the N-glycan profile. The levels of all types of N-glycans were lower (by ~4-fold) in 2-DG-treated GSC11 cells than in control cells. In contrast, N-glycan synthesis in GSC11 cells could be rescued to almost "normal control" levels by adding exogenous D-mannose. The rescue of 2-DG-treated GSC11 cells by D-mannose drastically reduced the incorporation of 2-DG into the N-glycans. These results indicate that 2-DG can interfere with biochemical transformations of D-mannose and that such interference might contribute to the overall antitumor effects of 2-DG.

394: Efficient and Stereoselective Synthesis of Alpha-(2,9) Oligosialic Acids Up to Dodecamer by Using Convergent Block Synthetic Strategy

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The vaccines against meningococcal group C diseases have been widely used. However, it is difficult to understand the optimal epitope because the vaccines are constructed by α(2→9) polysialic acids which are isolated from natural sources and often heterogeneous and/or contaminated with other antigenic components. In order to understand the structure and activity relationship of commercial meningitis vaccines, scientists have tried to develop a effective method to synthesize pure α(2→9) oligosialic acids with well defined structure in recent years. Convergent block synthesis is a straightforward and efficient strategy to synthesize oligomer or polymer. However, in the case of oligosialic acids, this strategy is particularly challenging since poor a-selectivity has been frequently observed when glycosylation used di/oligosialic acid unit as a glycosylation donor. Here, we demonstrate that α(2→9) dodecasialic acid derivative could be synthesized in 12 steps by the use of our developed 5-N,4-O-carbonyl protected phosphate-based donors[1] via the convergent block synthetic strategy. The success of convergent [4+8] strategy is significant because the a-selectivity retains even when the size of donor or acceptor increases. We believe that this method should be applied to the synthesis of higher oligomers, and our research in using these synthesized polysialic acid with homogeneous structures to construct polysialic acid-protein conjugate vaccine is underway.

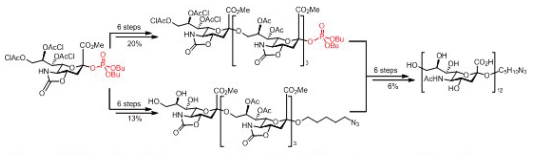

Figure: α(2,9) dodecasialic acid has been stereoselective synthesized in 12 steps via convergent block synthetic strategy.

Reference: [1] C.-H. Hsu, K.-C. Chu, Y.-S. Lin, J.-L. Han, Y.-S. Peng, C.-T. Ren, C.-Y. Wu, and C.-H. Wong, Chem. Eur. J., 2010, 16, 1754-1760.

395: Withdrawn

396: Synthesis of Mycobacterial Phosphatidylinositol Mannosides

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Mycobacterium tuberculosis (Mtb), an acid-fast Gram-positive bacterium that persists in infected macrophages for a long-term period, is responsible for the development of the active disease, tuberculosis (TB). Although the current BCG vaccine is effective for preventing the incidence of miliary TB in children, it is not effective at protecting adults from TB infection. Due to the spread of multidrug-resistant Mtb strains and the increase of patients with lethal HIV-TB coinfection, the development of new drugs or vaccines to combat TB is indeed important. The Mtb cell envelope, consisting of outer capsule, cell wall, and plasma membrane, is responsible for the initial stage of host cell infection. It forms a thick protective barrier to prevent antibiotic penetration and manipulates the host immune system to develop a favorable environment for bacterial survival and growth. The covalently ester-linked mycolic acids and peptidoglycan–arabinogalactans are the major components of cell wall, while phosphatidylinositol D-mannosides (PIMs), lipomannan (LM), lipoarabinomannan (LAM), and mannose-capped lipoarabinomannan (ManLAM) are non-covalently attached to the plasma membrane or outer capsule through their phosphatidylinositol anchor containing palmitic, stearic, and tuberculostearic acid residues. Among these components, PIMs are ubiquitously distributed in both pathogenic and non-pathogenic species of mycobacteria and play significant roles in cell-wall biogenesis and in many immunomodulatory events, including neutralization of potentially cytotoxic O_2 free radicals, induction of cytokines, phagocytosis of organism by binding with the mannose receptors, and growth of organism in the host macrophages. In continuation of our interest in the biosynthesis of PIMs and the development of anti-TB vaccines, we herein report a straightforward method for the synthesis of PIMs.

397: Synthesis of Glycopeptide Standards - Molecular Tools for Quantitative Glycoproteomics

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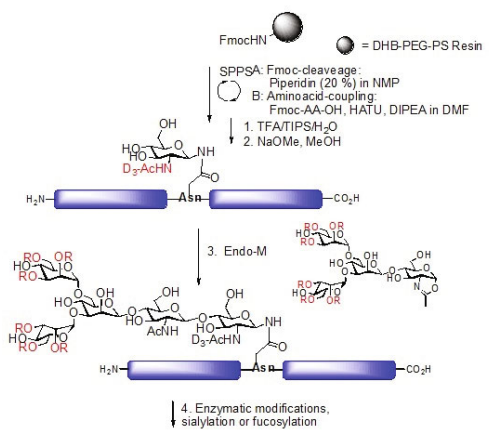
Many proteins are co- or posttranslationally modified by mono- or oligosaccharides. It has become evident that these glycoproteins play important roles in diverse biochemical processes. Quantitative proteomics is a valuable tool to follow directions of glycosylation, which generate new knowledge for basic understanding of glycosylation processes as well as their function in disease. Synthesis of N- and O-glycopeptide standards could facilitate precise quantification in glycoproteomics using mass spectrometry. Such standards include both stable isotope labelled glycopeptides function as internal standards or non-labelled glycopeptides as external standards. Synthetic glycopeptides are also valuable as model substances to better understand mass fragmentation behaviour as well as to improve methods of enrichment/ purification of different compound classes. Protein N-glycosylation sites on blood platelets have been extensively studied by the proteomics group at our institute [1], in order to further explore N-glycosylation on the glycopeptide level, synthetic glycopeptides are prepared. Synthetic methodology employing a chemoenzymatic endoglycosidase (endo M) coupling of N-glycan oxazoline donors and N-GlcNAc glycopeptides is the most promising approach for effective preparation of N-glycopeptides [2]. The current work to prepare various N-glycan oxazoline donors will here be presented in further detail. Oxazoline donors with different N-glycan structures could also be valuable tools in preparation of N-glycopeptides/ glycoproteins to further explore structure activity relationships or conformation effects of different N-glycosylation.



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398: Streamline One-Pot Protection of Carbohydrates Using Free Sugars

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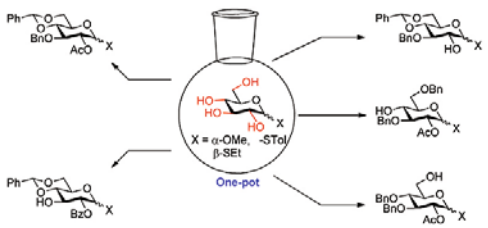
The assembly of oligosaccharides is accomplished by chemo- and stereo-selective coupling of monosaccharide units, and therefore relies on the monosaccharide building blocks bearing unprotected hydroxyl group at different position and orthogonal protecting groups that can be manipulated under different conditions. However, the preparation of these indispensable building blocks is still a very laborious endeavour without common intermediate and methodology. To differentiate each hydroxyl and introduce proper protecting groups at appropriate positions, various routes involving lengthy protection-deprotection sequences with tedious workups and purifications after each single step are necessary. Here, we report a streamline one-pot protection of carbohydrates directly using free sugars. Its applications to glucosamine and α,α'-D-trehalose are also described.



399: 3-Fluoro-1-hydroxyacetone Phosphate (FHAP) – A Novel Donor Substrate in Rabbit Muscle Aldolase-Catalyzed Aldol Reactions

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Rabbit muscle aldolase (RAMA) catalyzes the stereospecific aldol condensation of various aldehydes with dihydroxyacetone (DHAP), reliably forming two stereocenters with d-threo configuration. Due to RAMA's versatile tolerance of acceptor aldehydes, the enzyme became an outstanding catalyst for the formation of carbohydrates and related natural products.(1) However RAMA is very restricted concerning the nucleophile donor DHAP.(2)

Herein we report the development of an efficient gram scale synthesis of FHAP and demonstrate its application as a novel donor substrate for RAMA-catalyzed aldol condensations. FHAP is the first analog of DHAP with a modification at C3 accepted as a donor substrate by RAMA. A fast proton-deuterium exchange for one of the two enantiotopic protons at C3 of FHAP was analyzed by 1H-

and ^{19}F-NMR. Furthermore the binding preference for the keto form over the gem-diol form of FHAP was proved by ^{19}F-NMR, indicating that the FHAP is similar positioned in the active site as DHAP. FHAP allows the straightforward synthesis of 3-deoxy-3-fluoro-uloses, valuable probes for studying carbohydrate processing enzymes in biological system by ^{19}F-NMR.

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400: Pseudaminic and Legionaminic Acid: Synthesis of Sialic Acid Analogues

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Sialic acids and sialic acid analogues belong to one of the most challenging class of carbohydrates, which are mainly found in vertebrates.[1] They are located at the terminal ends of glycoproteins, glycolipids, phospholipids and proteoglycans and therefore influence many cell-cell and cell-molecule interactions, but also host-pathogene interactions are thought to be regulated via these molecules.[2] The common structural motif of these compounds consists of a nine carbon ketose backbone containing a deoxy position at C3 and a carboxyl group at C1. Different substitution patterns with oxygen and nitrogen functionalities offer many variations among this class of carbohydrates. The development of sialic acids is considered to happen mainly in mammalian cells, but various bacteria have also evolved their own sialic acid like carbohydrates such as pseudaminic or legionaminic acid.[3] Although their biological function is undeniable, only few synthetic protocols exist that offer access towards these rare molecules. We herein present a versatile methodology starting from threonine using a nitroaldol reaction for chain elongation and subjecting the resulting aldehyde to an indium mediated Barbier type reaction, which introduces the C1 to C3 segment of these sugars. The synthesis allows a variaty of both stereochemical and structural modification within the carbon backbone.

References: [1] R. Schauer; Glycoconj. J. 2000, 17, 485-499; [2] R. Schauer; Curr. Opin. Struct. Biol. 2009, 19, 507-514; [3] X. Chen, A. Varki; ACS Chem Biol 2010, 5, 163-176.

401: Synthesis of neoglycoconjugates containing 4-amino-4-deoxy-L-arabinose (Ara4N) ligands of *Burkholderia cepacia* and *Proteus mirabilis* R45 inner core lipopolysaccharides

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4-Amino-4-deoxy-L-arabinose (Ara4N) substitution of LPS is a frequently detected modification in Gram-negative bacterial strains that have developed resistance to cationic antimicrobial peptides (CAMP) via reduction of the anionic surface charge. Ara4-N modification has been reported for the lipid A domain and the inner core region, wherein Ara4N either is linked to the 8-position of a 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) unit (as in *Proteus mirabilis* R45) or at O-8 of a distal 3-hydroxy-analogue of Kdo, termed Ko, in *Burkholderia cepacia*.[1,2] To assess the epitope specicifities of antibodies binding to the inner core region of B. cepacia and P. mirabilis LPS, BSA neoglycoconjugates containing Ara4N-Ko/Kdo ligands have been prepared. Starting from 8-O-tBDMSi allyl glycosides of Ko and Kdo, respectively, an N-phenyltrifluoroacetimidate donor of Ara4N was coupled to suitably protected acceptors followed by global deprotection. Spacer groups were introduced via 1,3-propanedithiol addition to the anomeric allyl group, to be subsequently efficiently coupled to maleimide-activated BSA. This way, the BSA conjugates containing the disaccharide ligands Ara4N-(1→8)-Kdo, Ara4N-(1→8)-Ko as well as the branched trisaccharide Ara4N-(1→8)-[Kdo-(2→4)]-Kdo have been synthesized in a highly convergent approach.

Acknowledgments: Financial support from FWF (grants P 19295 and P 22909).
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2. Y. Isshiki, K. Kawahara, U. Zähringer, Carbohydr. Res., 1998, 313, 21-27.

402: Synthetic *Francisella* lipid A – based neoglycoconjugate

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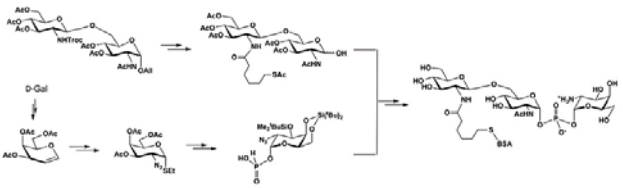
The lipopolysaccharide (LPS) of *Francisella*, an extremely virulent gram-negative pathogen having high potential for bioterrorism, has many unique structural properties. An important role in the pathogenesis of *Francisella* LPS plays its tetraacylated lipid A, which lacks the 4´-phosphate and is covalently modified with α-linked galactosamine at the single phosphate moiety at position 1 of the reducing glucosamine. [1-3] Absence of galactosamine substitution in attenuated live vaccine strains (LVS) suggests its potential role in virulence. Around 90% of *Francisella* Lipid A is present in the "free" form which lacks the usual Kdo, core, and O-antigen and presumably is substituted with α-linked glucose at the 6´ position. [4,5] The lipid A-based neoglycoconjugate containing chemically synthesised epitope β-D-GlcN-(1→6)-α-D-GlcN-

(1→P→1)-α-D-GalN, which is conserved in all virulent *Francisella* strains, was prepared by coupling of its thiol terminated spacer to maleimide – activated BSA. The newly synthesised *Francisella* lipid A based neoglycoprotein serves as a candidate for the generation of specific antibodies in an attempt to elaborate effective sub-unit vaccine. [6] In addition, immobilization of the epitope on gold surfaces enables its utilization in diagnostic immunoassays as capture antigen.



Financial support from FWF (grant P 21276) is gratefully acknowledged.

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403: Synthesis of Mucin Tandem Repeat Glycopeptides - Molecular Tools to Study Microbe Binding Preferences

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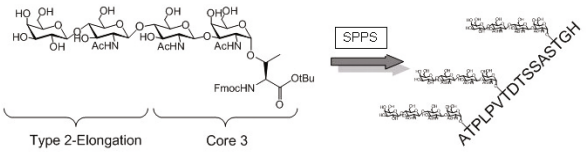
The Mucin glycoproteins, which all have heavily glycosylated tandem repeat peptide regions rich in PTS residues, are the major constituent of the mucus layer. This layer is a part of the innate immune system, and function as a protective barrier against invading pathogens in epithelial tissues[1]. Changes in the expression level and glycan structures of different mucins effect the physical (flow and viscosity) properties of the mucus. In chronic airway diseases (asthma, cystic fibrosis, COPD) an overproduction of mucin occurs, together with changes in the proportion between the different mucins and their glycan structures[2]. These structural changes are inflammatory-infective responses and contribute to defect flow properties of the mucus, which further induce a chronical inflammatory status and an environment where microbes (*Pseudomonas aeuroginosa, Staphylococcus aureus, Streptococcus pneumonia, Haemophilus influenza*) can grow and thrive instead of being cleared from the system.

In order to study the role of mucin glycosylation in mechanisms of infection, mucin tandem repeat glycopeptides will be prepared and employed in microbe binding studies. A library of mucin tandem repeat glycopeptides consisting of the secreting mucins MUC5AC and MUC5B and the membrane bound mucins MUC1 and MUC4 that are expressed in the airways, will be synthesized. The glycopeptides will be prepared employing type 1 or type 2 extended core 1-4 glycosylated amino acid building blocks and other Fmoc protected amino acids. Furthermore the synthesized glycopeptides will be modified with fucosyl- and/or sialyl- and/or sulfonyltransferases or further extended with GlcNAc and Gal-tranferases.



Scheme : Example for a type 2 elongated core 1 amino acid in solid phase peptide synthesis (SPPS).

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404: Muc1: a/ß-Hybrid glycopeptides as potential anti-cancer vaccines

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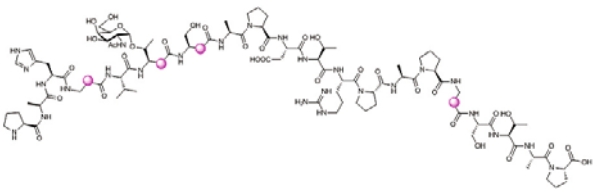
The membrane bound glycopeptide MUC1 decorated with tumor-associated carbohydrate antigens (TACA) is extensively overexpressed on epithelial tumor cells and is considered an important structure for the development of moleculary defined anti-cancer vaccines. [1] The limited metabolic stability of the glycopeptide conjugates constitutes one of the major challenges for the development of efficient carbo-hydrate-based vaccines. A strategic incorporation of ß-amino acid building blocks into mucin-type sequences might offer the potential to create pseudo-glycopeptide antigens with improved bioavailability. Towards this end, three TACAs (T_N, TF and ST_N antigen conjugates) O-glycosidically linked to Fmoc-ß^3-homo-threonine were prepared via Arndt-Eistert homologation of the corresponding glycosyl α-amino acid derivative. Furthermore first mixed hybrid (glyco)peptides were synthesised to investigate the stability towards different proteases as well as the change in enzymatic kinetics. [2]



References: [1] Taylor-Papadimitriou, J. et al. Trends Biotechnol. 1994, 12, 227–233; Danishefsky, S. J. et al. Angew. Chem. Int. Ed. 2000, 39, 836–863; Slovin, S. S. et al. Immunol. Cell Biol. 2005, 83, 418–428. Becker, T.et al. Curr. Opin. Mol. Ther. 2007, 9, 35–44. Liakatos, A. et al. Curr. Opin. Mol. Ther. 2007, 9, 35–44; Buskas, T. et al. Chem. Commun. 2009, 5335–5349. [2] Karch, F., Hoffmann-Röder, A., Beilstein J. Org. Chem., 2010, 6, No. 47; Norgren, A. S.; Norberg, T.; Arvidsson, P. I. J. Pept. Sci. 2007, 13, 717–727.

405: Synthesis of mucin type photoluminescent nanoprobes

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Mucins are heavy glycosylated membrane-associated or secreted glycoproteins that play important biological roles in the ocular, respiratory, reproductive and intestinal tracks.[1] Structural changes of the O-glycan chains have been linked to changes in gene expression due to diseases such as inflammatory bowel disease, cystic fibrosis and gastrointestinal and breast type cancers, and may also reflect regional lymph node metastasis.[2, 3] Despite their importance, little is known about the specific role of mucins in both healthy and diseased states and this is due mainly to the incomplete knowledge of mucin's structural composition. The efficient synthesis of well defined fragments of this class of glycans, ready to be immobilized into carbohydrate arrays for high-throughput biological analysis, is crucial to help us understand the biological and disease implications of these glycosylation patterns at both molecular and functional levels. In the context of cancer associated glycan epitopes, we report a highly convergent strategy for the preparation of Core 1-4 mucin type glycans that contained an α-linked aminopropyl spacer for direct attachment to glycoarray platforms. A two-directional approach is used whereby consecutive orthogonal glycosylation reactions can be accomplished with complete chemo- and stereo- selective control.

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406: UniCarbKB: Building Glycoinformatic Solutions for Glycomics Research

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UniCarbKB is a new initiative to create a universal glycomics knowledgebase built on collections of experimental, structural and functional data. The project continues to build on the success of recent glycobioinformatic frameworks by extending the availability and application of databases and tools.The provision of an information rich central hub is a crucial resource and integral part of glycomics discovery that will enhance data interpretation, and data storage for the dissemination of knowledge generated from current glycomic research strategies. Knowledge of the structure and function of glycoproteins is vital and we introduce activities to deploy a comprehensive, curated and publically accessible database of glycan structures and protein information. An important part of this program is the integration of structural, literature, and biological source information that is publically available in GlycoSuiteDB into the UniCarbKB portal, along with highly annotated structural data sourced from EUROCarbDB. The portal will be central for collecting data on structures released from specific protein/tissues and associated changes in glycosylation due to disease, essential to understanding the functional roles of glycans.

A key part of this development is UniCarb-DB (www.unicarb-db.org) an experimental component of the knowledgebase that expands on the efforts of EUROCarbDB. It addresses the lack of an informatic infrastructure to support the growing trend for LC-MS approaches that are vital to large-scale glycomic and glycoproteomic studies. Here, we present the first database release of oligosaccharide structures and fragment data characterized by LC-MS/MS techniques. The database is annotated with high-quality data sets and is designed to extend and reinforce those standards and ontologies developed by existing initiatives. Its availability will serve as a base for the development of new analytical tools for structural data querying and MS spectra interpretation. By providing access to high-quality experimental data and cross-referencing to a curated structure database this tool can become a vital resource for analyzing glycomic data.

407: Withdrawn

408: Glycoanalysis of Carbohydrate-associated Epitope(s) Recognized by RP215 Monoclonal Antibody

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Glycoanalysis was performed to elucidate the carbohydrate-associated epitope(s) specific to RP215 monoclonal andtibody, which was initially generated against OC-3-VGH ovarian cancer cell extract. MALDI-TOF MS analysis of tryptic peptides was employed to identify the molecular identity the corresponding CA215 antigen which was affinity-isolated from the shed medium of cultured cancer cells. The results revealed that CA215 consists of glycoproteins including immunoglobulin superfamily (IgSF) proteins (~60%), mucin (~7%) and other related ones, each of which contains RP215-specific epitope(s) expressed preferentially among cancer cells, but rarely in normal human tissues. Experiments were performed to elucidate the glyco-structures of this unique epitope(s). Profilings of O-linked and N-linked glycans, glycosylation site mappings as well as linked glycoanalysis were carried out with CA215 isolated from both OC-3-VGH ovarian and C33A cervical cancer cells. For N-linked glycans, high mannose and complex bisecting structures were detected with the terminal N-glycolylneuraminic acid (NeuGc). In the case of O-linked glycans, several oligosaccharides with structures similar to those of mucins, but with terminl NeuGc which are rarely found in human IgG. Identical O-linked glycan profiles were obtained from CA215 isolated from either cancer cell lines. O-linked and N-linked glycosylation mappings of CA215 revealed the identification of two N-linked and eight O-linked glycopeptides. Protein blast search for peptide sequence homology indicated that two N-linked and six O-linked glycopeptides were almost 100% matched to immunoglobulin heavy chains. The results of these studies seemed to suggest that both N-linked and O-linked glycans with unique terminal NeuGc in CA215 are structurally related to those of mucins and may be involved in the epitope recognitions by RP215.

409: Integration of LC, MS and NMR for a sensitive and Fast Characterization of Oligosaccharides from Glycoproteins

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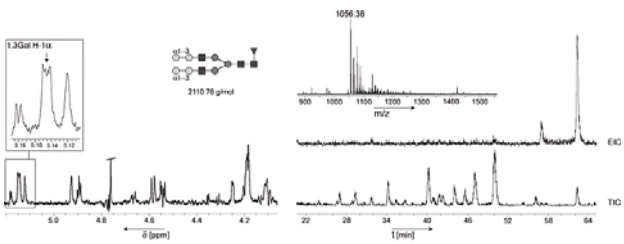
Despite a lot of progress analysis of glycan structures from glycoproteins is still time consuming. A new tight integration of LC, MS, and NMR proves very powerful for the fast analysis of glycan mixtures. The integration presented here eliminates the problems of each method, such as signal overlap in NMR spectra or the lack of information on stereo centers in MS. Oligosaccharides from glycoproteins are often obtained as mixtures in very limited quantities. MS is thought to be of much higher sensitivity than NMR. However, we could characterize just 15 pmol (30 ng) of an N-type complex decasaccharide by NMR spectroscopy using the structural reporter group concept. Here, we present the integration of LC, MS, and NMR for the analysis of glycan mixtures, where the individual components were present from 200 pmol (400 ng) to 900 pmol (1.8 ug) each. The mixtures contained up to five complex carbohydrates. All components could be identified by integration of NMR and MS. Stereo isomer and the quantity of individual glycan structures were derived from NMR and the MW from MS. As a test we also analyzed glycan structures of bovine fibrinogen, which yielded more than 20 structures as a mixture. By combining LC, MS and NMR we were able to characterize new structures that have not been reported. As example, we present spectra of a biantennary oligosaccharide carrying the highly immunogenic α1,3-linked galactosyl residues (publ. in prep.). Based on these data we are working on a tighter integration of LC, MS, and NMR.



Data on the glycan shown on top carrying two a1,3 Gal residues. Left: NMR spectra and right: MS data. The NMR spectrum is shown for the anomeric structural reporter group region with an inset highlighting the signals for a1,3 Gal. The MS base peak chromatogram and the extracted ion chromatogram for the doubly charged ion (top) is shown.

410: Serum alpha 1-acid glycoprotein is a marker for the differential diagnosis of hepatitis B patients

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Aberrant glycosylation of serum protein has been known to be associated with pathogenesis of various human diseases including hepatic diseases. Hepatitis B and hepatitis C caused by hepatitis B virus (HBV) and hepatitis

C virus (HCV) due to latency and without intervention turn to liver cirrhosis and further to liver cancer. In certain pathophysiological condition N-glycosylation pattern of the acute-phase protein, viz., alpha-1 acid glycoprotein (AGP) undergoes changes. The aim of the present study is to give detail mass spectrometric analysis to ascertain the changes in oligosaccharides of AGP from sera of the patients suffering from chronic hepatitis B (HBV-CH) and hepatitis B induced liver cirrhosis (HBV-LC) with respect to healthy individuals. AGP from the sera of patients and healthy individuals was purified by affinity chromatography on monoclonal anti-AGP sepharose 4B column. N-glycans released from AGP by hydrazinolysis were labeled with 2-aminopyridine (2-PA). The labeled oligosaccharides were separated by three successive HPLC columns, viz., diethylaminoethyl (DEAE), octadecyl silica (ODS) and amide silica. The structure of oligosaccharides was determined by HPLC mapping method in conjunction with mass spectrometry. Our results show that in both HBV-CH and HBV- LC patients' serum AGP remarkable decrease in sialic acid and increase in galactose residues were observed, however, their relative quantity was found to be different in two types of patients.

411: Determination of the fine structure of oat arabinogalactan-protein and its ability to bind to human leucocytes

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An arabinogalactan-protein (AGP) from whole grain of oat (*Avena sativa* L.) has been isolated for the first time by double precipitation with β-glucosyl Yariv reagent and characterized with regard to its polysaccharide and protein part with common methods like neutral sugar analysis, linkage analysis, size exclusion chromatography and amino acid analysis. Additional treatment with mild acid hydrolysis and alkali treatment are conventional methods so get further insights in the polysaccharide structure. The specific hydrolysis with compatible recombinant enzymes (exo-β-1,3-galactanase and exo-α-1,5-arabinofuranosidase [1,2]) with subsequential structure determination of the remaining macromolecule and HPAEC-PAD of the released mono- and oligosaccharides are qualified methods to gain further information on the fine structure of the complex polysaccharide part of the AGP from oat [3]. Additionally, a binding study of the oat AGP to human leucocytes was carried out, with regard to its putative immunomodulating activity [4]. References: [1] Ichinose H et al. (2005) J Biol Chem 280(70): 25820-25829. [2] Ichinose H et al. (2008) Appl Microbiol Biot 80(3): 399-408. [3] Goellner EM et al. (2011) J Cereal Science, in press. [4] Thude S et al. (2006) Phytomed 13: 425-427.

412: Oligosaccharide from *Ganoderma lucidum* polysaccharide: immune response in human peripheral blood mononuclear cell

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Oligosaccharide, molecular weight 1-5 kDa, was found to have impressive immune-property as the native *Ganoderma lucidum* polysaccharide, molecular weight around 500 kDa. This oligosaccharide was degraded by Smith degradation of the *Ganoderma lucidum* macromolecule and elucidated the sugar sequence by MS and NMR spectroscopy as $[-\alpha\text{-}1,4\text{-Glc-}(\beta\text{-}1,4\text{-GlcA})_3\text{-}]_n$. A fraction of proteoglycan with molecular weight above 20 KDa was also obtained by size-exclusion chromatography. The native *Ganoderma lucidum* polysaccharide, proteoglycan, and oligosaccharide can induce the CD69 expression of monocytes, NK cells, and T lymphocytes in human peripheral blood mononuclear cells. The separated oligosaccharide displayed the specific immune property of low monocyte induction, and greatly stimulated cell activation and proliferation of NK and T cells. This glucuronic acids/glucose oligosaccharide from *Ganoderma lucidum* polysaccharide may respond for the active stimulation of NK and T cells.

413: Bis(monoacylglycero)phosphate: Direct Determination of the Diglycerophosphate Backbone Configuration

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Bis(monoacylglycero)phosphate (BMP), also widely know as lysobisphosphatidic acid (LBPA), is a minor constituent of most animal tissues. It is highly enriched in lysosomes, the recycling centre of eukaryotic cells. Low density lipoprotein (LDL) containing cholesteryl esters, foreign antigens and excess nutrients among others are delivered to lysosomes and subsequently degraded. It has been show that BMP greatly stimulates the lysosomal enzymes involved in the degradation of glycosylceramides and that BMP is as a key player to maintain cholesterol homeostasis. Consequently, BMP is involved in the pathology of lysosmal storage diseases, like Niemann-Pick Type C, sphingolipidosis, glycoprotein and glycogen storage diseases. Despite of its biological importance, the biosynthetic pathway of BMP is yet unknown. In 1967, Body and Gray reported that BMP is a structural isomer of phosphatidylglycerol (PG), featuring two glycerol subunits linked via a phosphate

diester. To-date, no direct spectroscopic evidence of the stereochemical configuration of the diglycerophosphate backbone has been reported. As a first step we determined the stereochchemical configuration of the BMP backbone, utilizing d-camphor ketals as a chiral NMR shift reagent. For comparison, we synthesized all three possible diglycerophosphate backbone analogues of BMP. In order to clarify the biosynthetic pathway of BMP, we are currently on route towards stable isotope labelled PG analogues, a know precursor of BMP. Starting from prochiral [$^{13}C_3$]glycerol, we prepared enantiomerically pure 3-para-methoxybenzyl protected [$^{13}C_3$]glycerol as a key intermediate in three steps, based on our experience with glycerol d-camphor ketals.

414: Why do side chains of flax fiber rhamnogalacturonan I have lower mobility than the backbone?

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Understanding the biological role of carbohydrates requires a complete characterization not only of its structure but also of spatial organization and molecular dynamics. We tried to adapt the diffusion-ordered NMR spectroscopy for characterization of complex polysaccharide macromolecular packing. Rhamnogalacturonan I of the gelatinous cell walls from flax phloem fibers was used as the analyzed polymer. The backbone of this polysaccharide is built as RG I; the attached to C4 of rhamnose long side chains consist mainly of β-1,4-galactose and have variable structure. Determination of the diffusion coefficients revealed two groups of signals on rhamnogalacturonan I DOSY spectra. The first group included the signals of the polymer backbone – H1, H2, H3, H6,6' of branched rhamnose; H1, H2, H3, H4, H6,6' of unbranched rhamnose and signals of single galactose residue (H2, H4). The diffusion coefficient of these signals is twice higher than of the second group, which included mainly the signals of linear galactose (H1, H2, H3, H4). We suggest that the difference in mobility of the flax rhamnogalacturonan I backbone and side chains is the evidence of interactions between the side chains. The lower mobility of the chains as compared to the backbone might be expected only if the interaction is provided by the side chains, which form the core of the complex, while the backbone is located at the periphery. The shape of such intermolecular complexes may be spherical or cylindrical. Removal of some of the side chains and part of the backbone from the formed complex would not change the hydrodynamic volume, which determines the elution time during gel filtration. This was confirmed by gel-filtration of the polymer before and after treatment with the specific glycanases. The proposed macromolecular organization of rhamnogalacturonan I is highly relevant to function of this polymer in the gelatinous secondary cell wall, where it plays a key role in tension creation.

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