Appl Biochem Biotechnol (2010) 162:1435–1445
DOI 10.1007/s12010-010-8909-0

Expression and Immunogenicity of a Recombinant Chimeric Protein of Human Colorectal Cancer Antigen GA733-2 and an Fc Antibody Fragment in Stably Transformed *Drosophila melanogaster* S2 Cells

Yeon Ju Seok · Kyung Il Kim · Ki Hyun Yoo · Jeon Hwang-Bo · Hyun Ho Lee · Dong Hwa Shon · Ki Sung Ko · Hyung Sik Kang · Kyung Jin Lee · Doo-Byoung Oh · Young Hee Joung · In Sik Chung

Received: 30 October 2009 / Accepted: 4 January 2010 /
Published online: 30 January 2010

Abstract Human colorectal cancer antigen GA733-2 fused to the immunoglobin Fc fragment (GA733-2-Fc) was expressed in stably transformed *Drosophila melanogaster* S2 cells, and the immunogenicity of recombinant GA733-2-Fc was investigated in mice. Recombinant GA733-2-Fc was secreted into a culture medium with a molecular mass of approximately 65 kDa. Recombinant GA733-2-Fc was purified to homogeneity using affinity fractionation with Protein A sepharose 4 Fast Flow. Recombinant GA733-2-Fc proteins elicited production of specific antibodies against recombinant GA733-2 by immunization through an intraperitoneal route. Recombinant GA733-2-Fc-induced antibodies showed a binding activity to human colorectal carcinoma HCT-116 cells. Secretory recombinant GA733-2-Fc from *Drosophila* S2 cell systems can be used as an effective experimental antigen for research in cancer vaccine development.

D. H. Shon
Food Immunology Research Group, Korea Food Research Institute, Seongnam-si, Kyunggi-do 463-746, Korea

K. S. Ko
Department of Life Science, WonKwang University, Iksan 156-756, Korea

H. S. Kang · Y. H. Joung
School of Biological Science and Technology, Chonnam National University, Gwangju 500-757, Korea

K. J. Lee · D.-B. Oh
Integratve Omics Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 111 Gwahangno, Yuseong-gu, Daejeon 305-806, Korea

Y. J. Seok · K. I. Kim · K. H. Yoo · J. Hwang-Bo · H. H. Lee · I. S. Chung (✉)
Department of Genetic Engineering and Graduate School of Biotechnology, Kyung Hee University, Suwon 446-701, Korea
e-mail: ischung@khu.ac.kr

Keywords Cancer antigen · *Drosophila melanogaster* S2 cells · Expression · Immunogenicity · Recombinant GA733-2-Fc

Introduction

The GA733-2 antigen is a 40 kDa transmembrane glycoprotein that functions as a homotypic cell-cell adhesion molecule. The GA733-2 gene encodes a 314-amino acid polypeptide that includes a 23-amino acid signal sequence, a 242-amino acid extracellular domain, a 23-amino acid transmembrane domain, and a 26-amino acid cytoplasmic domain. The extracellular domain of GA733-2 antigen has three potential *N*-linked glycosylation sites and an N-terminal cystein-rich region containing 12 cysteines [1]. The extracellular domain of antigen GA733-2 has been used as a target for cancer vaccination in several clinical trials. When used as a recombinant protein in cancer patients, tumor cell-specific humoral and cellular immune responses were observed [2]. Currently, the vaccinia virus is used to deliver GA733-2 to cancer patients in ongoing clinical trials [3].

Expression of recombinant GA733-2 has been reported in expression systems including baculovirus-infected Sf21 cells [4], *Drosophila melanogaster* S2 cells [5], and plant cell systems [6, 7]. Sera from mice immunized with recombinant GA733-2 from baculovirus-infected insect cell systems show a binding activity to SW1116 colorectal carcinoma cells [4]. Recombinant GA733-2 proteins produced in *Drosophila* systems have also been used for detection of auto-antibodies [5]; however, the in vivo immunogenicity of recombinant GA733-2 derived from *Drosophila* systems in mice has not been reported.

Stability of heterologous peptides has been achieved through diverse strategies such as modification with polyethylene glycol (PEGylation) or by fusion of peptides with the immunoglobin Fc domain [8, 9]. Chimeric fusion of heterologous peptides with the Fc fragment may improve stability, and in some cases, increase antigen immunogenicity [10]. In this study, human colorectal cancer antigen GA733-2 fused to the Fc fragment of the antibody (GA733-2-Fc) was expressed in stably transformed *D. melanogaster* S2 cells. Also, the immunogenic response of recombinant GA733-2-Fc in mice was investigated to examine its potential use in cancer immunotherapy research.

Materials and Methods

Cell Lines and Expression Plasmids

D. melanogaster Schneider 2 (S2) cells were grown at 27°C in a T-25 culture flask (Nunc, Roskilde, Denmark) in Shields and Sang M3 Insect Medium (Sigma, St. Louis, MO, USA) containing 10% (*v*/*v*) IMS (Insect Medium Supplement; Sigma). The 3.6 kb pMT/BiP/V5-His plasmid (Invitrogen, USA) contained a metallothionein promoter, a BiP signal sequence, a V5 epitope tag, and a polyhistidine region. The selection plasmid pCoHygro (Invitrogen), which contained the bacterial hygromycin B phosphotransferase gene under control of the constitutive *Drosophila* Copia 5-LTR promoter, was used for stable transformation. Human colon carcinoma HCT-116 cells were maintained in RPMI-1640 medium (Hyclone, USA) supplemented with 10% FBS and 100 units/ml penicillin-streptomycin (Invitrogen, USA). HCT-116 cells were cultured in a T-25 culture flask in a humidified environment at 37°C in the presence of 5% CO_2. *E. coli* DH5α was used as the primary host for constructing and propagating plasmids. *E. coli* cells were routinely grown

in shake flasks at 37°C with agitation at 200 rpm in LB medium [1% tryptone, 0.5% yeast extract, and 0.5% NaCl (pH7.3)] containing 50 mg/ml of ampicillin. DNA restriction enzymes from Promega (USA) and Takara (Japan) were used according to the manufacturer's instructions.

Construction of Expression Plasmids

Human GA733-2 cDNA (NM_002354) was amplified from total RNA extracted from human colon carcinoma HCT-116 cells using reverse transcription-PCR with oligonucleotide primers. The sense primer was 5′-AAGCTTGATACGGCGACTTTTGCC-3′ and the antisense primer was 5′-GCGGCCGCTTTTAGACCCTGCATTGAG-3′. The Fc fragment of human IgG cDNA (Gene Bank accession no. AY172957) was amplified from the total RNA extracted from human B cells IM-9 (Korean Cell Line Bank, Korea) using reverse transcription-PCR with oligonucleotide primers. The sense primer was 5′-GCGGC CGCAGATCTCGTTGAGCCCAAATCTTGTG-3′ and the antisense primer was 5′-GCGGCCGCACCCGGGGACAGGGAG-3′. Amplified human GA733-2 cDNA and human Fc sequences were inserted into the pGEM-T vector (Promega) to yield pGEM-T/GA733-2 and pGEM-T/Fc, and the sequences were verified by DNA sequencing. GA733-2 (the *Hind*III and *Not*I fragment of pGEM-T/GA733-2) was then fused to the Fc fragment of human IgG (the *Not*I fragment of pGEM-T/hFc) to yield pBluscript II SK(+)/GA733-2-Fc. GA733-2-Fc and GA733-2 cDNAs were amplified from the pBluscript II SK(+)/GA733-2-Fc plasmid vector using PCR with oligonucleotide primers. For PCR of GA733-2-Fc cDNA, the sense primer was 5’- CCATGGGATACGGCGACTTTTGCC-3’ and the antisense primer was 5′-TCTAGAACCCGGGGACAGGGAG-3′. For PCR of GA733-2 cDNA, the sense primer was 5’-AGATCTACGGCGACTTTTGCCGC-3’ and the antisense primer was 5′-CTCGAGTTTTAGACCCTGCATTGAG-3′. The amplified GA733-2-Fc and GA733-2 sequences were also inserted into the pGEM-T vector (Promega) to yield pGEM-T/GA733-2-Fc and pGEM-T/GA733-2, and the sequences were verified by DNA sequencing. PCR was performed using a Thermal Cycler (PE Biosystems, USA) with PCR Mix (Takara) in a volume of 50 μl. pMT/BiP/GA733-2-Fc-V5-His was constructed by inserting the *Nco*I–*Xba*I fragment of pGEM-T/GA733-2-Fc between the *Nco*I and *Xha*I sites of pMT/Bip/V5-His. As a control, pMT/BiP/GA733-2-V5-His was also constructed by inserting the *Bgl*II–*Xho*I fragment of pGEM-T/GA733-2 between the *Bgl*II and *Xho*I sites of pMT/BiP/V5-His. The proper orientation and reading frame of the insertions in pMT/BiP/GA733-2-Fc-V5-His and pMT/BiP/GA733-2-V5-His were confirmed using both restriction enzyme mapping and DNA sequencing.

Stable Transformation and Analysis of Recombinant GA733-2-Fc Expression

Exponentially growing S2 cells were co-transfected with the pMT/BiP/GA733-2-Fc-V5-His and pCoHygro plasmids (5:1) using the lipofectamine method as described elsewhere [11]. For preparation of the transfection medium, plasmid DNA and the lipofectamine reagent (Invitrogen) were diluted separately in IMS-free M3 medium, and then mixed at a 1:5 ratio. The transfection medium was incubated at room temperature for 45 min and then transferred to 6-well plates pre-seeded 2 h earlier with S2 cells in IMS-free M3 medium. After a 24-h incubation, the medium was changed to remove the lipofectamine, and the cells were incubated for an additional 5 days without drug selection in M3 medium containing 10% IMS. The cells were then centrifuged and resuspended in selective M3 medium containing 10% IMS and 300 μg/ml hygromycin B (Duchefa,

Haarlem, Nertherlands). The selective medium was replaced every 5 days and stably transformed polyclonal cell populations were isolated after 4 weeks of selection with hygromycin B, which was maintained continuously in the medium after the selection procedure.

As a control, exponentially growing S2 cells were also co-transfected with the pMT/BiP/GA733-2-V5-His and pCoHygro plasmids (5:1) using the lipofectamine method as described above. Stably transformed S2 cells expressing either GA733-2-Fc or GA733-2 were cultured for 5 days in Nunc (T-25) flasks to analyze expression of GA733-2-Fc or GA733-2, which was induced by addition of 0.5 mM $CuSO_4$ after the start of the run.

Purification of Recombinant GA733-2-Fc

All steps were performed at 4°C. Recombinant GA733-2-Fc was purified using affinity fractionation with Protein A sepharose 4 Fast Flow (GE Healthcare, Sweden) according to the manufacturer's recommendations. Briefly, the medium fraction of the culture was applied to a Protein A sepharose 4 Fast Flow column. Weakly bound contaminating proteins were washed from the beads using the binding buffer (20 mM sodium phosphate buffer, pH7.0). Recombinant GA733-2-Fc proteins were then eluted with 0.1 M glycine (pH3.0) and dialyzed in phosphate buffered saline (PBS, pH 7.4). Also, recombinant GA733-2 and GA733-2-Fc were purified using Ni-NTA resin (Qiagen, Valencia, CA, USA) in a chromatography column (Novagen, Madison, WI, USA) as described elsewhere [12]. Protein concentrations were determined using a Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA) with BSA as a standard.

Deglycosylation of Recombinant GA-733-2-Fc

Purified recombinant GA733-2 was digested using *N*-glycosidase (PNGase) F and *O*-glycosidase (Roche, Basel, Switzerland). PNGase F can cleave all types of *N*-glycans bound to aspargaine residues of proteins except the glycans containing α(1,3)-fucose attached to the innermost GlcNAc. *O*-glycosidase releases the Gal β(1-3) GalNAc unit from *O*-glycans, which is bound to either serine or threonine as a core unit. For reaction with one of the enzymes, 5 μg of purified recombinant GA733-2 was deglycosylated for 18 h at 37°C with either 1 U of PNGase F in 50 mM sodium phosphate buffer (pH7.5) or 1.5 mU of *O*-glycosidase in 50 mM sodium acetate buffer (pH 5.0). For reaction with both enzymes, 5 μg of purified recombinant GA733-2 was treated with a cocktail of 1 U of PNGase F and 1.5 mU of *O*-glycosidase in 50 mM sodium phosphate buffer (pH7.5) for 18 h at 37°C. Reactions were stopped by addition of SDS sample buffer. Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described previously.

N-glycan Analysis

Purified GA733-2-Fc was first digested into glycopeptides with 0.1 μg of pepsin in 10 mM HCl buffer (pH2.2) for 12 h at 37°C and incubated for an additional 12 h after the addition of the second batch of 0.1 μg pepsin. From the resulting glycopeptide mixtures, *N*-glycans were released using peptide *N*-glycosidase (PNGase) A (Roche, Basel, Switzerland), which can cleave all types of *N*-glycans including the plant- and insect-specific glycans attached with core α(1,3)-fucose. The purification of the cleaved glycans was carried out as previously described [13]. After deglycosylation, the obtained glycans were labeled using

8-amino-1,3,6-pyrenetrisulfonic acid (APTS, Molecular Probes, Carlsbad, CA) and then analyzed on a ABI 3130 DNA sequencer (Applied Biosystems, Foster City, CA, USA) according to a previously reported protocol [13]. The APTS-labeled glycans in 20 mM ammonium acetate buffer at pH5.3 were treated with exoglycosidases including bovine kidney α-fucosidae, *Aspergillus saitoi* α(1,2) mannosidase, and jack bean *N*-acetylhexosaminidase (Prozyme, Hayward, CA, USA) for their structural analysis as previously described [13].

Immunization of Animals

This study protocol was approved by the Institutional Animal Care and Use Committee of Kyung Hee University, and animal care and experimental procedures followed university guidelines for the care and use of laboratory animals. Six-week-old female BALB/c mice (ten mice/group) were divided into four groups and immunized intraperitoneally four times at 2-week intervals with (a) recombinant GA733-2-Fc (45 μg) plus adjuvant, (b) recombinant GA733-2 (30 μg) plus adjuvant, (c) PBS plus adjuvant, and (d) PBS only. An amount of 30 μg of GA733-2 was estimated to be the same molar amount as 45 μg of the fusion protein GA733-2-Fc. As an adjuvant, complete Freund's adjuvant and incomplete Freund's adjuvant were used in an emulsified from for the first immunization and for additional boosting immunizations, respectively. Blood samples were collected from mouse tails 3 days before the first immunization (day3), a week after each immunization, and at 3-day intervals from 1 week after the last immunization. Sera were separated and stored at −70 °C until use.

Assay of Antibody Binding to the Recombinant Antigen

Binding of serum antibodies from immunized BALB/c mice to recombinant GA733-2-Fc antigens was determined using an enzyme-linked immunosorbent assay (ELISA). In brief, the purified recombinant GA733-2 from transformed *Drosophila* S2 cells was coated on 96-well ELISA plates at a concentration of 200 ng/well. After an overnight incubation at 4 °C, the plates were washed three times with PBS-T (PBS, pH7.4 containing 0.05% Tween-20) and then blocked for 1 h with assay diluent (PBS, pH7.4 containing 1% BSA) at room temperature. After washing with PBS-T, mouse antiserum serially diluted in an assay diluent was placed on the plates and incubated for 1 h. The plates were further washed and incubated for 1 h with goat anti-mouse IgG-horseradish peroxidase conjugate (1:5,000 dilution in an assay diluent, Jackson ImmunoResearch, USA). After washing with PBS-T, the plates were developed for 30 min using tetramethylbenzidine substrate solution containing hydrogen peroxide, and then stopped by addition of 2 M H_2SO_4. The absorbance was measured at 450 nm using an ELISA microplate reader [8].

Assay of Antibody Binding to Cancer Cells

To determine binding of serum antibodies from immunized mice to human colorectal cancer cell lines, ELISA plates were coated with HCT-116 (1×10^4 cells/well) and incubated overnight at 37 °C. After fixation in 50 μl of 0.05% glutaraldehyde in PBS for 20 min at room temperature, cells were washed once with PBS, and then blocked with 50 μl of 0.7% glycine. Serum samples from immunized mice were diluted to 1:100 and incubated in the wells for 1 h. The rest of the procedure for the assay, following treatment with the antisera, was the same as described above.

Results and Discussion

Expression of Recombinant GA733-2-Fc

Expression of recombinant GA733-2-Fc in stably transformed S2 cells was analyzed using Western blot analysis at 5 days after induction with 0.5 mM $CuSO_4$. The recombinant GA733-2-Fc protein (a molecular mass of approximately 65 kDa) was detected in transformed S2 cells carrying pMT/BiP/GA733-2-Fc-V5-His using Western blot analysis with mouse anti-V5 polyclonal antibodies (data not shown). This molecular mass is higher than predicted molecular mass (58.6 kDa) for the recombinant protein. On the basis of the vector construct, cleavage of the BiP signal peptide yielded a mature GA733-2-Fc protein of 522 amino acids that contained the 487 amino acids of GA733-2-Fc, the 23 amino acids of the C-terminal tag of V5 and His_6, and 12 amino acids of extra sequences derived from the cloning sites. The mature protein was a product of further glycosylation before secretion. Recombinant GA733-2-Fc was present in both the intracellular and extracellular (medium) fractions of transformed S2 cells. Densitometric scanning showed that the secreted GA733-2-Fc (i.e., GA733-2-Fc in the medium) accounted for approximately 71% of total GA733-2-Fc production. The stably transformed S2 cells efficiently secreted recombinant GA733-2-Fc into the medium. Recombinant GA733-2-Fc was not detected in either the cellular or medium fractions of non-transfected S2 cells.

Recombinant GA733-2-Fc was rapidly purified to near homogeneity using a protein A sepharose affinity purification procedure. The purity of the protein was analyzed using SDS-PAGE and silver staining (Fig. 1a). Western blot analysis further confirmed the identity of the purified protein (Fig. 1b). Amounts of 29.6 μg and 22.7 μg of purified GA733-2-Fc protein were obtained by purification using Protein A sepharose 4 Fast Flow and Ni-NTA resin, respectively, from the medium fraction of a 30 ml culture. No visible contaminating proteins were observed on silver nitrate-stained SDS-PAGE gel. Thus, the separation efficiency of the method using Protein A sepharose 4 Fast Flow was improved by 30% compared with the efficiency of the method using Ni-NTA resin. We believe that Fc-tag can be used as an alternative to His-tag for single-step purification of recombinant proteins from other expression systems. The assembly of recombinant GA733-2-Fc proteins under non-reducing condition was determined using SDS-PAGE, confirming multimeriza-

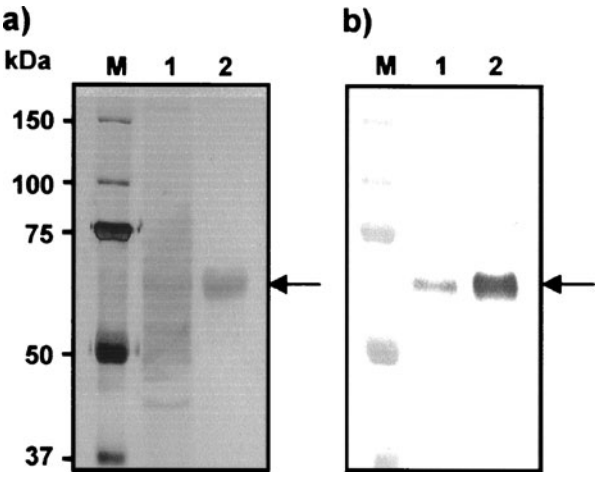

Fig. 1 SDS-PAGE (**a**) and Western blot analysis (**b**) for purification of recombinant GA733-2-Fc from the medium fraction of a stably transformed S2 cell culture. *M* pre-stained molecular size marker, *1* pre-binding sample, *2* 1 M imidazole elution

tion (data not shown). The ability of these molecules to multimerize might confer some structural advantage in terms of recombinant protein stability expressed in *Drosophila* S2 cells. The assembly status of the chimeric peptide has been suggested to be a determinant of stability for the recombinant HIV-1 p24-immunoglobulin fusion molecule [10].

Deglycosylation of recombinant GA733-2-Fc using glycosidases and re-analysis on SDS/PAGE were performed to examine the reduction in apparent molecular mass (Fig. 2). The apparent molecular mass for purified recombinant GA733-2-Fc was approximately 65 kDa. Recombinant GA733-2-Fc digested using PNGase F and *O*-glycosidase resulted in a single band of approximately 59 kDa. The results shown in Fig. 2 demonstrate that recombinant GA733-2-Fc does not contain any *O*-linked glycans, which can be removed by *O*-glycosidase. Analysis of the chimeric GA733-2-Fc peptide sequence using the NetNGlyc 3.1 server (http://www.cbs.dk/services) and the OGPET 1.0 server (http://ogpet.utep.edu/OGPET) indicates that the chimeric GA733-2-Fc protein has three potential sites (N^{63}, N^{100}, and N^{342}) for *N*-linked glycosylation, but does not contain any consensus sites for *O*-linked glycosylation.

To elucidate the *N*-glycan structure of recombinant GA733-2-Fc, glycan analysis based on the DNA sequencer technique was performed [13]. The resulting APTS-labeled glycan profiles are shown in Fig. 3 and the peaks were identified primarily by exoglycosidase digestion. The biggest peak, designated as trimannosyl core glycan attached with a fucose residue ($Man_3GlcNAc_2Fuc_1$), dominates 48%. Oligommannose glycans ($Man_{5\sim9}GlcNAc_2$) accounts for 36% while paucimannose ($Man_2GlcNAc_2Fuc_1$) and trimmanosyl core glycan without fucose (Man3GlcNAc2) constitute for 10% and 6%, respectively.

The attached fucose residues were easily removed using bovine kidney α-fucosidase at relative low concentration, which indicates that the fucoses would be linked to core GlcNAc via an α(1,6)-glycosidic linkage because core α(1,3)-fucose is relatively resistant to α-fucosidase [14, 15]. The glycans obtained using PNGase F digestion displayed the same profile as the glycans obtained using PNGase A (data not shown). It has been reported that *Drosophila* core α(1,3)-fucosyltransferase prefers α(1,6)-fucosylated glycans as a substrate and that most of α(1,3)-fucosylated glycans are also α(1,6)-fucosylated [15]. However, peaks corresponding to bifucosylated glycans were not found in the glycan profile. α(1,3)-fucose linked to innermost GlcNAc of *N*-glycan, which was not observed in mammals, has been regarded as a potentially allergic epitope [16]. Our result indicates that

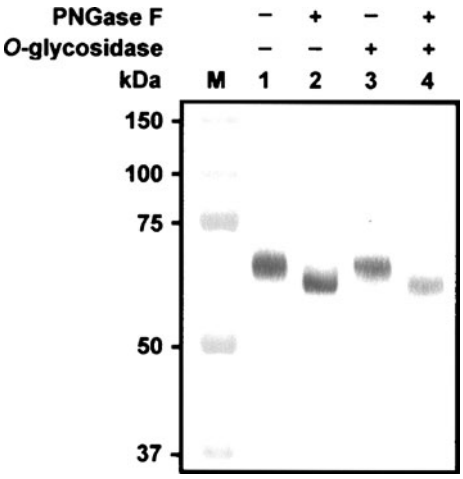

Fig. 2 Deglycosylation of recombinant GA733-2-Fc. Purified recombinant GA733-2-Fc was treated with PNGase F (*lane 2*), *O*-glycosidase (*lane 3*), and with a cocktail of PNGase F and *O*-glycosidase (*lane 4*). *Lane M*, molecular mass markers

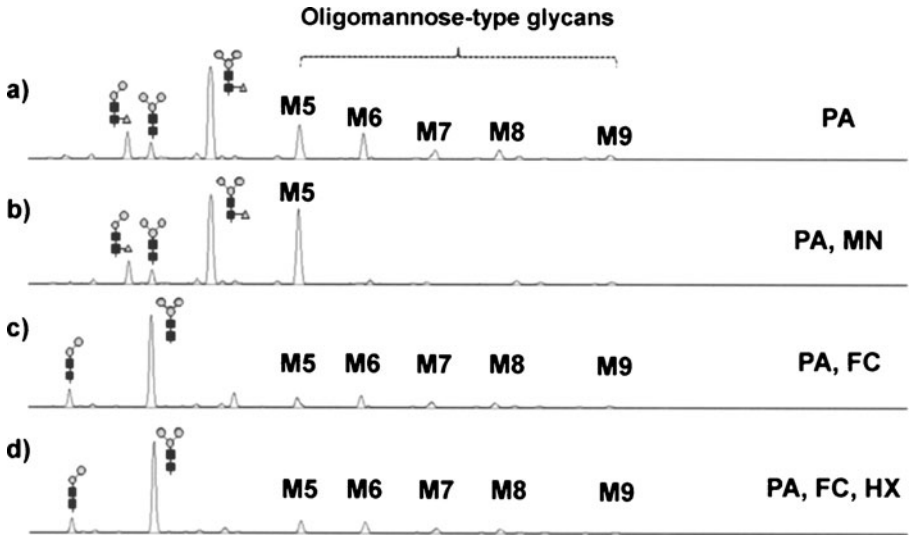

Fig. 3 *N*-glycan profiles of GA733-2-Fc. **a** The profile of APTS-labeled glycans was obtained using PNGase A (*PA*) digestion. **b**–**d** The glycans peaks were further analyzed using exoglycosidase digestions, including *A. saitoi* α(1,2)-mannosidase (*MN*), bovine kidney α-fucosidase (*FC*), and jack bean *N*-acetylhexosaminidase (*HX*). Symbols used for glycans structures are those suggested by the Consortium for Functional Glycomics (http://www.functionalglycomics.org). *Filled circles* mannose, *filled squares N*-acetylglucosamine (GlcNAc), *filled triangles* fucose. M5~9 indicate oligomannose-type glycans ($Man_{5\sim9}GlcNAc2$)

recombinant GA733-2-Fc from *Drosophila* S2 cell systems does not contain the core α(1,3)-fucose. This point may be an advantage in use of *Drosophila* S2 cell systems for manufacture of an effective experimental antigen for research in cancer vaccine development.

Immunogenicity of Recombinant GA733-2-Fc in Mice

To investigate specific antibody production, antisera from BALB/c mice immunized with either the GA733-2 or GA733-2-Fc recombinant proteins were applied to ELISA plates

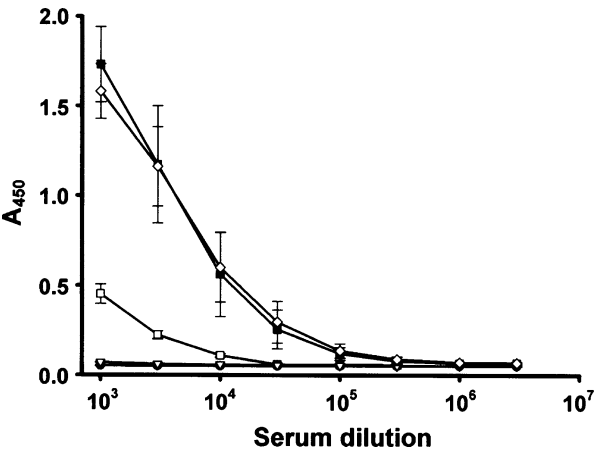

Fig. 4 GA733-2-specific IgG antibodies in sera from mice immunized i.p. with recombinant either GA733-2-Fc or recombinant GA733-2. Blood samples were collected from immunized Balb/c mice 1 week after the final immunization. Serum samples were serially diluted and titrated using ELISA. *Filled circles* PBS, *empty squares* PBS+FA, *filled squares* GA733-2+FA, *empty diamonds* GA733-2-Fc+FA, *empty squares* mouse anti-EpCAM mAb (Santa Cruz). Data represent the mean±SD from ten mice per group

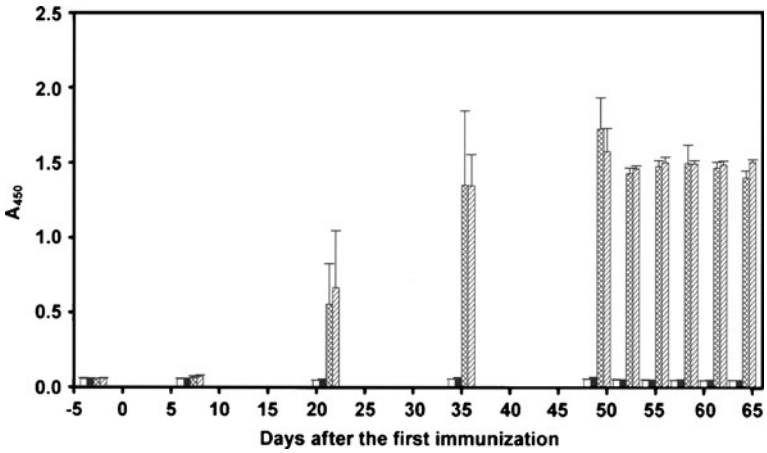

Fig. 5 Change of anti-GA733-2 specific IgG antibodies in sera from mice immunized i.p. with either recombinant GA733-2-Fc or recombinant GA733-2. Balb/c mice were immunized i.p. four times at 2-week intervals with either recombinant GA733-2-Fc or recombinant GA733-2. Serum samples were collected 3 days before the first immunization, 1 week after each immunization, and at 3-day intervals beginning 1 week after the 4th immunization. Serum samples were diluted to 1:1,000 and analyzed using ELISA. □ PBS, ■ PBS+FA, ▩ GA733-2+FA, ▨ GA733-2-Fc+FA. Data represent the mean±SD from ten mice per group

coated with recombinant antigen GA733-2. ELISA values for anti-recombinant GA733-2-Fc antisera were much higher than values for commercial EpCAM mAb (Santa Cruz), and were similar to values for anti-recombinant GA733-2 antisera (Fig. 4). Therefore, recombinant GA733-2-Fc-induced specific antibody production with high titers in mice and had almost the same potential to elicit antibodies against GA733-2 as recombinant GA733-2. It has been reported that fusion of the antigen with the IgG Fc fragment may

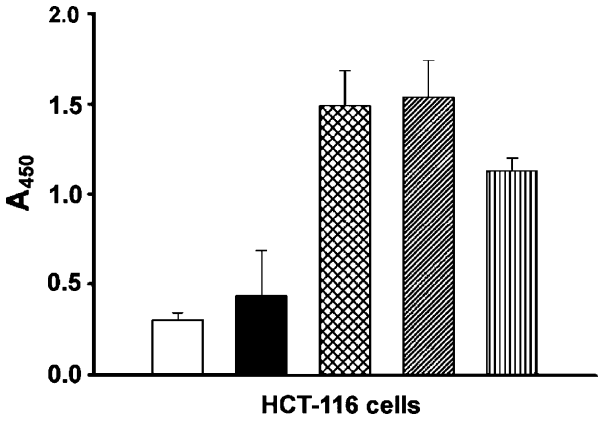

Fig. 6 Reactivity of serum samples from immunized mice on GA733-2 positive colon cancer HCT-116 cells. Blood samples were collected from immunized BALB/c mice 1 week after the final immunization, and diluted to 1:100 for ELISA analysis. □ PBS, ■ PBS+FA, ▩ GA733-2+FA, ▨ GA733-2-Fc+FA, ▥ mouse anti-EpCAM mAb (Santa Cruz). Data represent the mean±SD from ten mice per group

increase the immunogenicity of the antigen [10]. However, in this study, recombinant GA733-2-Fc did not show any Fc effects in the antibody response against GA733-2. Spitsin et al. [17] reported no detectable fusion-related enhancement of immunogenicity or quality of the immune response for fusion of the avian flu antigen with the IgG Fc fragment.

A time-course change for anti-GA733-2-Fc IgG antibody production in immunized mice showed that the titer of the specific antibodies in the antisera increased gradually due to immunizations, attained a high level after the third immunization, and reached the highest level after the fourth immunization. Then, the titer remained relatively constant until the 65th day after the first immunization (Fig. 5). The time-course pattern for anti-GA733-2 IgG antibody production was similar to the pattern for anti-GA733-2-Fc IgG antibody production.

To confirm reactivity of sera samples from immunized mice on GA733-2 positive colon cancer cells, in vitro cell ELISA was performed. Mouse serum samples were diluted and applied to ELISA plates coated with human colorectal carcinoma HCT-116 cells. Recombinant GA733-2-Fc-induced antibodies showed higher binding activities to human colorectal carcinoma HCT-116 cells than commercial EpCAM mAb (Santa Cruz) (Fig. 6). Fc effects cannot be entirely excluded, but are unlikely considering the similar binding activities of the antibodies raised against both the GA733-2 and the GA733-Fc antigens for HCT-116 cells. Antibodies produced by immunization with the recombinant GA733-2-Fc protein were highly reactive with human colorectal carcinoma cells. These antibodies should be the subject of research regarding their anti-colon cancer immunotherapeutic potential.

To the best of our knowledge, this is the first report of in vivo immunogenicity of the recombinant chimeric protein (GA733-2-Fc) of human colorectal cancer antigen GA733-2 and the Fc antibody fragment from *Drosophila* S2 cell-expression systems. The Fc antibody fragment of the chimeric GA733-2-Fc protein allowed the purification efficiency to be improved by 30% using the protein A-based method, compared to the Ni-NTA resin-based method. It appears that fusion of heterologous peptides with the human immunoglobin Fc fragment facilitates the purification process in different expression systems. The ability of fusion molecules containing the Fc fragment to assemble together probably confers some structural advantage for protein stability. In addition, the glycosylated and highly charged Fc fragment enhances the solubility of hydrophobic proteins [18]. Our findings also show that secretion and functional expression of recombinant chimeric protein (GA733-2-Fc) in *Drosophila* S2 cell systems provides a convenient source of recombinant colorectal cancer antigens for research into cancer vaccine development.

Acknowledgement This work was supported by a grant from the Biogreen 21 Project (20070401034026).

References

1. Chong, J. M., & Speicher, D. W. (2001). *Journal of Biological Chemistry, 276*, 5804–5813.
2. Maruyama, H., Zaloudik, J., Li, W., Sperlagh, M., Koido, T., Somasundaram, R., et al. (2000). *Cancer Immunology, Immunotherapy, 49*, 123–132.
3. Kim-Schulze, S., Kim, H. S., Wainstein, A., Kim, D. W., Yang, W. C., Moroziewicz, D., et al. (2008). *Journal of Immunology, 181*, 8112–8119.
4. Strassburg, C. P., Kasai, Y., Seng, B. A., Miniou, P., Zaloudik, J., Herlyn, D., et al. (1992). *Cancer Research, 52*, 815–821.
5. Schmetzer, O., Moldenhauer, G., Riesenberg, R., Pires, J. R., Schlag, P., & Pezzutto, A. (2005). *Journal of Immunology, 174*, 942–952.

6. Verch, T., Hooper, D. C., Kiyatkin, A., Steplewski, Z., & Koprowski, H. (2004). *Cancer Immunology, Immunotherapy, 53*, 92–99.
7. Brodzik, R., Spitsin, S., Golovkin, M., Bandurska, K., Portocarrero, C., Okulicz, M., et al. (2008). *Cancer Immunology, Immunotherapy, 57*, 317–323.
8. Nie, Y., Zhang, X., Wang, X., & Chen, J. (2006). *Bioconjugate Chemistry, 17*, 995–999.
9. Lee, T.-Y., Sjin, R. M., Movahedi, S., Ahmed, B., Pravda, E. A., Lo, K.-M., et al. (2008). *Clinical Cancer Research, 14*, 1487–1493.
10. Obregon, P., Chargelegue, D., Drake, P. M., Prada, A., Nuttall, J., Frigerio, L., et al. (2006). *Plant Biotechnology Journal, 4*, 195–207.
11. Lee, J. M., Lee, H. H., Hwang-Bo, J., Shon, D. H., Kim, W., & Chung, I. S. (2009). *Biotechnology and Applied Biochemistry, 53*, 101–109.
12. Lee, J. M., Hwang-Bo, J., Sohn, B. H., & Chung, I. S. (2007). *Protein Expression and Purification, 52*, 258–264.
13. Lee, K. J., Jung, J. H., Lee, J. M., So, Y., Kwon, O., Callewaert, N., et al. (2009). *Biochemical and Biophysical Research Communications, 380*, 223–229.
14. Kubelka, V., Altmann, F., Staudacher, E., Tretter, V., Marz, L., Hard, K., et al. (1993). *European Journal of Biochemistry, 213*, 1193–1204.
15. Fabini, G., Freilinger, A., Altmann, F., & Wilson, I. B. H. (2001). *Journal of Biological Chemistry, 276*, 28058–28067.
16. Tomiya, N., Narang, S., Lee, Y. C., & Betenbaugh, M. J. (2004). *Glycoconjugate Journal, 21*, 343–360.
17. Spitsin, S., Andrianov, V., Pogrebnyak, N., Smirnov, Y., Borisjuk, N., Portocarrero, C., et al. (2009). *Vaccine, 27*, 1289–1292.
18. Lo, K.-M., Sudo, Y., Chen, J., Li, Y., Lan, Y., Kong, S.-M., et al. (1998). *Protein Engineering, 11*, 495–500.