Chapter 13

The Role of ELR$^+$-CXC Chemokines in Wound Healing and Melanoma Biology

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1. MGSA/GRO Is a Member of the Chemokine Superfamily of Chemotactic Cytokines

Chemokines are small proinflammatory peptides that regulate trafficking, activation, and sometimes the proliferation of myeloid, lymphoid, melanocytes, keratinocytes, and endothelial cells *(1)*. The chemokines have been divided into four subfamilies based upon structure and function: the CXC, CX_3C, CC, and C chemokines *(2–4)*. The CXC chemokine family includes four MGSA/GRO (melanoma growth stimulatory activity/growth-related oncogene) genes (α,β,γ,δ) as well as interleukin 8 (*IL-8*), gamma interferon-inducible gene (*IP-10*), monocyte induced by γ-interferon (*MIG*), *ENA-78*, granulocyte chemotactic protein-2 (*GCP-2*), neutrophil activating peptide-2, the mitogen for B-cell progenitors known as stromal derived factor-1 (*SDF-1*), and others *(2,5–14)*. The proteins encoded by these genes exhibit an NH_2 terminal cysteine alignment of two cysteines separated by an intervening amino acid (CXC) *(2,15–17)* (*see* Table 1). The CXC chemokines that contain an ELR motif at the amino terminus are angiogenic (*IL-8*, *MGSA/GRO*, *NAP-2*, *ENA-78*, *GCP-2*), whereas those not containing this motif are angiostatic (*MIG*, *IP-10*, *PF-4*) *(18)*. The murine *MGSA/GRO* orthologs are *KC* and *MIP-2*. The chemokine-β subfamily, noted by two adjacent cysteines (CC) at the N terminus, includes RANTES, MCP-1–3, MIP-1α and β, and numerous others *(2,19)*. Only one γ-chemokine has been identified, lymphotactin, and this chemokine is characterized by a single conserved cysteine in the amino terminus of the protein *(3)*. Lymphotactin is expressed in progenitor T-cells and is chemotactic for lymphocytes but not monocytes or neutrophils. Fractalkine is the single CX_3C-chemokine identified thus

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Table 1
CXC Chemokines and CXC Receptors

CXC Chemokines		CXC Chemokine Receptors		
Human	Mouse	Rat	Human	Mouse
MGSA/GROα	KC	CINC	CXCR2	mCXCR2
MGSA/GROβ	MIP-2α	MIP-2	ECRF3 + KHV8 – GPCR	"
MGSA/GROγ	MIP-2β		DARC	"
IL-8			CXCR1 + CXCR2 + ECRF3 + DARC	
ENA-78	ENA-78		CXCR1 and 2	
GCP-2	GCP-2		CXCR1 and 2	
NAP-2			CXCR2 + ECRF3 + KHV8 – GPCR	
IP-10	IP-10		CXCR3	mCXCR3
MIG			CXCR3	
SDF	SDF		CXCR4	mCXCR4
PF4			KHV8-GPCR	

far and this chemokine has three intervening amino acids between the first two cysteine residues *(4)*. This chemokine is expressed on endothelial cells and promotes the adhesion of monocytes as well as T cells. The expression of various members of the chemokine superfamily is regulated by cytokines, growth factors, and agents such as LPS, phorbol ester, and glucocorticoids *(2,13,20–32)*.

2. ELR$^+$ CXC Chemokine Receptors

The biological functions described above for the CXC chemokines are mediated through specific, shared, and promiscuous receptors. Two different CXC receptors were identified by expression cloning from neutrophil RNA *(33,34)* (Table 1). CXCR1 binds IL-8 and GCP-2 with high affinity and MGSAα, β, γ, ENA-78, and NAP-2 with much lower affinity (approx 30–100-fold lower) *(35,36)*. CXCR2 has high affinity for both IL-8 and MGSA/GROα, β, and γ, and also binds three other CXC chemokines, NAP-2, GCP-2, and ENA-78 *(35–38)*. The genes for these two receptors are located on human chromosome 2 *(39)* and the 42–44 kDa proteins encoded by these genes may be glycosylated in neutrophils producing receptors sized at approx 58–65 kDa *(40,41)*. Only one CXC receptor has been published from mouse and this receptor binds both KC and MIP-2 with high affinity *(42–45)*. CXCR3 is the receptor for *MIG* and IP-10, whereas CXCR4 (fusin) is the receptor for the CXC chemokine, SDF-1 *(46)*. Viruses also encode CXC-chemokine receptors. For example, the Herpesvirus saimiri, HVS-ECRF3, is a virally encoded shared CXC chemokine receptor that binds MGSA/GRO, IL-8 and NAP-2 *(47,48)* and the promiscuous Kaposi's HHV8 G-protein-coupled receptor binds IL-8, NAP-2, PF-4, MGSA/GRO as well as the CC chemokines RANTES and I309 *(49)*. Analysis of the predicted protein structure of these receptors suggests that these distinct receptors, along with 10 recently cloned C-C chemokine receptors *(50)*, are members of the G-protein-coupled, seven-transmembrane domain receptor family *(51)*. MGSA/GRO, NAP-2, IL-8, RANTES, and MCP-1 exhibit high affinity binding to the Duffy antigen receptor for chemokines (DARC), a promiscuous seven-transmembrane chemokine receptor that serves as the point of entry for *Plasmodium vivax (47,52–54)*.

MGSA/GRO will block *P. vivax* binding to DARC and thus block erythrocyte invasion by this malaria-causing organism *(52,54)*. It has been proposed that this receptor might function to clear plasma IL-8 and MGSA/GRO during the acute-phase response *(55)*. It is not clear whether the erythrocyte receptor recycles in a manner similar to CXCR1 and CXCR2, but it does not couple to G proteins. However, DARC is sequestered in response to ligand. MGSA/GRO mutated in the E6 residue (substitution with alanine) binds human DARC and inhibits malaria invasion but does not signal through CXCR1 and CXCR2 *(56)*. There are in addition reports of novel receptors for MGSA/GRO *(57,58)*.

3. Biological Activities of MGSA/GRO Mediated Through CXCR2 and Other Chemokine Receptors

Although a number of chemokine-receptors bind MGSA/GRO, most of the biological functions of this chemokine are mediated through CXCR2. When expression of chemokines becomes disregulated resulting in chronic overexpression and chemokine-receptor activation, tissue damage *(59,60)*, angiogenesis *(61)*, and tumor growth can occur *(62–65)*.

3.1.1. Modulation of Chemotaxis and Growth/Differentiation of Hematopoietic Cells

A number of reports have demonstrated that MGSA/GRO, like IL-8, is chemotactic for neutrophils, basophils, monocytes, and lymphocytes *(34,37,38,66–71)*. Cerami's group has shown that the mouse form of the MGSA/GRO gene, MIP-2, works in combination with CSF to promote colony formation for myelopoietic progenitor cells (CFU-GM) *(7,72–76)*. Loss of the receptor for MIP-2 (CXCR2) results in alterations in the mechanisms for regulation of hematopoiesis *(77)*. Wang et al. have recently shown that MIP-2 induced a rapid mobilization of hematopoietic progenitor cells into peripheral blood in mice *(78)*. Mice lacking CXCR2 exhibit lymphadenopathy resulting from increased B cells, splenomegaly resulting from increased metamyelocytes, band, and mature neutrophils. The lymphadenopathy and splenomegaly were partially resolved when the mice were placed in a pathogen-free environment *(42)*. Though wound healing studies have not been performed on these mice, we would expect that the loss of keratinocyte and endothelial cell response to MIP-2 and KC should retard the wound healing and angiogenic responses to these chemokines during injury.

When synthetic MGSA/GRO was injected intradermally into mice at concentrations of 10^{-9} mol/site, there was a massive infiltration of neutrophils into the injected site *(79,80)*. The infiltration was most prominent around venules in the deeper dermal layers. Significant increases were also noted with decreasing concentrations of MGSA/GRO. The MGSA/GRO proteins as well as IL-8 affect the migration of T-lymphocytes, both $CD8^+$ and $CD4^+$ T-lymphocytes *(81,82)*. However, there is some controversy regarding the ability of CXC chemokines to activate $CD4^+$ T cells *(83,84)*. Apparently, cytokines such as IL-2, IFNγ and TNFα markedly upregulate the expression of the CXCR2 on T-lymphocytes and differential receptor expression could be the reason for the differing results from these two studies.

3.1.2. Inflammatory Disease Models Involving CXC Chemokines

Chemokines play a fundamental role in the host defense. The recruitment and activation of neutrophils, lymphocytes, and monocytes resulting from expression of MGSA/

GRO and other CXC chemokines with an ELR motif, is responsible for much of the tissue damage associated with chronic infection. Antibodies to MGSA/GRO as well as IL-8 will block this tissue damage by blocking leukocyte infiltration *(85–87)*. In rheumatoid arthritis, MGSA/GRO is remarkably elevated and accounts for one third of the neutrophil accumulation in RA joints, whereas IL-8 and ENA-78 contribute equally to this process based upon antibody-inhibition studies *(88)*. In rat, CINC, the rat MGSA/GRO homolog, has been implicated in immune complex glomerulonephritis and antibodies to CINC reduce 40–60% of the neutrophil infiltration and diminished proteinuria in response to GMB antibody-induced glomerular infiltration *(89)*. MGSA/GRO is elevated in persons with active ulcerative colitis *(90)*. MGSA/GRO proteins are among the major mediators of the adult respiratory distress syndrome *(87)*. The bronchoalveolar lavage fluid from patients with bacterial pneumonia have threefold higher levels of MGSA/GRO than IL-8 and antibody to MGSA/GRO has been shown to reduce the pulmonary inflammation induced by intratracheal administration of LPS by 71% *(86,87)*. Thus, in spite of studies showing IL-8 antibodies are effective in reducing the inflammatory infiltrate in ARDS, other CXC chemokines contribute to this inflammatory process. In the liver of alcoholics, chemokines are involved in the accumulation of neutrophils *(85)*. In a rat model of alcoholism, ethanol feeding is associated with increased production of MGSA/GRO/CINC and antibody to CINC blocks neutrophil accumulation in response to culture supernatants of hepatocytes isolated from ethanol feed rats *(85)*.

3.1.3. Chemokines in AIDS and Kaposi's Sarcoma

Chemokines and chemokine receptors play a pivotal role in AIDS pathogenesis. Both HIV-infected cells and Kaposi's sarcoma (KS) lesional cells express a high titer of chemokines *(91–96)*. Pneumonia patients with HIV infection show sevenfold higher MGSA/GRO than IL-8 in these fluids. The expression of chemokines MIP-1α, RANTES, MCP-1, and IL-8 by $CD8^+$ cells can have weak protective effects with regard to HIV infection of $CD4^+$ cells *(97–99)*. Levy has demonstrated that IL-8 can reduce viral entry for some strains, though it is not nearly effective as MIP-1α, MIP-1β, and RANTES *(100)*. IL-8 (100 ng/mL) inhibited HIV replication 50% in naturally infected $CD4^+$ cells, but only 20% in acutely infected $CD4^+$ cells. Lower concentrations (10 and 1 ng/mL) were not capable of significantly inhibiting virus replication. Antibodies to IL-8 would inhibit the effects of IL-8, but not the CAF produced by $CD8^+$ cells from HIV-resistant persons *(99)*. Recent data show that CXCR2 can function as a HIV-2 coreceptor in the fusion assay; when envelope proteins from HIV-2 are expressed on CHO fibroblast membranes these cells will fuse with cells expressing human CD4 and human CXCR2 *(101)*. These data suggest that virus entry is facilitated through the chemokine receptor. Consequently, if the chemokine receptor is blocked with the appropriate chemokine, then the virus can no longer bind and productively infect the cells. Use of chemokines in anti-HIV therapy should be approached with caution. Whereas expression of CXC chemokines might bring in a cascade of leukocytes to participate in the host response to infected cells, these chemokines may also bind to receptors on endothelial cells, stimulate angiogenesis, and thus participate in the growth of AIDS-associated malignancies. Many HIV-infected persons are also infected with *human herpesvirus-8* (HHV-8). The Kaposi's HHV8 G-protein-coupled receptor binds both CXC and CC chemokines. This receptor is naturally truncated at the C-terminal domain and when expressed in normal rat kidney epithelial cells is constitutively active, stimulates cellular proliferation, and thus may contribute to the growth of Kaposi's lesions *(49)*.

3.1.4. MGSA/GRO Expression Is Deregulated in Skin Diseases

CXC chemokines have been reported to stimulate the proliferation of keratinocytes in culture in combination with other mitogens *(69,102–104)*. In a large study of a variety of skin lesions, we compared the expression of MGSA/GRO in normal skin to that in seborrheic keratosis, actinic keratosis, keratoacanthoma, psoriasis, verruca (warts), skin tags, squamous-cell carcinoma, and basal-cell carcinoma *(60)*. In all the lesions, MGSA/GRO was expressed in the suprabasal keratinocytes and in the epidermal appendages of the skin such as hair follicles, sebaceous glands, sweat ducts, and in dermal blood vessels. However, two of the five normal skin samples were MGSA/GRO negative. The reasons for the variability of chemokine expression in normal controls remains an unsolved question. The lesions with the highest expression of MGSA/GRO were verruca (warts), followed by psoriasis, keratoacanthoma, and squamous-cell carcinoma. MGSA/GRO was also detected in sclerosing variants of basal-cell carcinoma but absent in the more common nodular variant. In keratinocytic lesions, MGSA/GRO expression correlated with the inflammatory response and degree of keratinocyte differentiation *(105)*. Melanocytic lesions stained less intensely for MGSA/GRO than keratinocytic lesions.

MGSA/GRO and IL-8 are overexpressed in psoriatic keratinocytes where these chemokines contribute to the ongoing inflammatory process and neutrophil infiltration associated with psoriasis *(59,60)*. Schroeder purified MGSA/GRO protein from psoriatic scales and demonstrated that this protein had neutrophil chemotactic activity *(106)*. These investigators also demonstrated that MGSA/GRO mRNA in the keratinocytes of the epidermal layers above the dermal papillae of psoriatic epidermis *(107)*. Gillitzer observed an upregulation of MGSA/GRO and IL-8 mRNAs in the upper epidermis of psoriatic keratinocytes. This expression of chemokine correlated with the neutrophil migration into the psoriatic lesions *(108)*. The receptor for MGSA/GRO, CXCR2, is overexpressed in keratinocytes of psoriatic lesions and receptor expression is diminished with therapy that resulted in decreased acanthosis *(109)*. MGSA/GRO and IL-8 are also involved in the homing of specific T cells in inflamed skin *(110)* and antibodies to CXCR2 will block the transendothelial migration of T cells expressing the cutaneous lymphocyte-associated antigen (CLA). Thus MGSA/GRO interaction with CXCR2 expressed in keratinocytes, T cells, and neutrophils appears to be a major contributor to psoriatic disease. Antagonist for CXCR2 might provide a highly effective theraputic approach for treatment of psoriasis.

3.1.5. Characterization of the Expression of CXCR2 in Skin Keratinocytes after Burn Injury

Three prior studies from other labs have implicated an important role for MGSA/GRO and related CXC chemokines in cutaneous wound healing. Two studies in rabbits following sulfur-mustard-induced burn lesions demonstrated increased expression of IL-8, MGSA/GRO, MCP-1, and IL-1 *(111,112)*. MGSA/GRO expression was upregulated in hair-follicle epithelial cells following sulfur-mustard treatment and chemokine expression was increased in the dermis providing a chemotactic gradient for recruitment of leukocytes and epithelial cells participating in wound repair. MGSA/GRO was postulated to facilitate autocrine-paracrine-mediated wound repair *(111,112)*. Explant lesions infiltrated by leukocytes exhibited chemotactic activity for PMN and released a number of chemoattractants, including IL-8. Rennekampff et al. have shown that blister fluids contain high levels of MGSA/GRO (0.79 ng/mL with a range of 0.018–4.86 ng/mL)

(113). Donor site wound fluids also contain levels of MGSA/GRO increasing from day 1 through day 5 and the level correlated with increasing levels of TNF. Addition of MGSA/GRO to cultured keratinocytes stimulated the growth 2.6-fold over 7 days and also stimulated an increase in the α6 integrin, but not in the α5, α2, or β1 integrins. Addition of topically applied MGSA/GRO (50 ng/cm2) on the healing of meshed split-thickness human skin grafts on athymic mice stimulated the rate of epithelialization ($p < 0.05$) at day 7 and increased the number of mitotic keratinocytes. MGSA/GRO treatment also reduced wound contraction, suggesting a role for MGSA/GRO in wound healing through the stimulation of the proliferation of keratinocytes *(113,114)*. This observation is in agreement with studies by Tuschil demonstrating that IL-8 stimulates epidermal cell proliferation *(103,113)*.

In the chicken model, the processed 9E3 protein, cCAF, has recently been shown to stimulate the growth of blood vessels in the chorioallantoic membrane assay (CAM). This event is accompanied by a thickening of the ectoderm of the CAM. The cCAF protein stimulates events associated with inflammation and granulation tissue formation in the absence of wounding *(115)*. Although chicken chemotactic and angiogenic factor (cCAF) has properties of both CC and CXC chemokines, its angiogenic and wound-healing activities suggest that, biologically, it behaves much as the chicken ortholog of MGSA/GRO. However, its chemotactic properties are more like CC chemokines in regard to monocyte/macrophage and lymphocyte recruitment *(116)*.

A role for CXC chemokines—especially those interacting with CXCR2—is clearly implicated in the cutaneous wound-healing model. We have correlated the expression of this receptor and its ligand during the wound-healing scenario *(105)*. The receptor is expressed in the migrating margins of the burn wound in proliferating keratinocytes involved in reestablishing the epidermis. Both the receptor and the MGSA/GRO ligand are also observed in the sweat glands, hair follicles, and in the endothelial cells undergoing neovascularization *(105,109)* (Tables 2 and 3). Expression of both mDARC and CXCR2 were observed in the endothelial cells of human burn wounds in areas of neovascularization. In granulation tissues, CXCR2 was noted in numerous fibroblasts and in subpopulations of macrophages and smooth muscle. Interestingly, the ligand is localized in the suprabasal keratinocytes, inner root-sheath cells, and dermal sweat ducts, whereas the receptor is found in both the epidermal and dermal compartments of healing wounds. In the dermis, polyvalent antibodies detected receptor immunoreactivity most prominently in the dermal sweat ducts, and in endothelial cells lining capillaries in the dermis. Receptor immunostaining was noted in migrating/proliferating keratinocytes in epithelial margins and islands, but was not detectable in the outer layers or in hypertrophic epidermis adjacent to wounds. The same pattern was observed in epidermal appendages such as hair follicles and eccrine sweat ducts. In the underlying granulation tissues, CXCR2 was noted in granulation tissue, in subpopulations of macrophages, and in smooth muscle. The presence of both MGSA/GRO and its receptor in human burn wounds implicate this cytokine as a possible autocrine or paracrine mediator of epidermal regeneration in both the inflammatory and proliferative phases of cutaneous wound repair *(105)*.

Additional verification of the expression of MGSA/GRO proteins in normal and wounded epidermis comes from in situ hybridization studies in which the antisense riboprobe vector that did not distinguish between the three forms of MGSA/GRO mRNA confirmed the expression of MGSA/GRO mRNA in these cells and tissues (J. Luan et al., unpublished data). We propose that MGSA/GRO within the suprabasal keratinocyte

Table 2
Immunodistributions in Normal Skin

Tissue	MGSA	MGSA Receptor
Epidermis		
Stratum basalis	–/–	++/+++
Stratum spinosum	++/+++	++/+++
Stratum granulosum	+/+++	++/+++
Stratum corneum	–	–
Epidermal appendages		
Eccrine sweat glands	+	–
Eccrine sweat ducts	++	+
Outer-root sheath/hair follicle	–/+	+/–
Inner-root sheath/hair follicle	+++	+/–
Sebaceous gland (peripheral cells)	–	+/–
Sebaceous gland (central cells)	+	–/–
Mesenchymal structures		
Endothelial cells	–	–/+/+++
Fibroblasts	–	+/–
Macrophages	–	++
Smooth muscle	++	–

(–) Not detectable; (+) faintly detectable; (++) moderately detectable; (+++) intense.
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Table 3
Immunodistributions Within Cutaneous Wounds

Tissue	MGSA	MGSA-R (419)	MGSA-R (415)
Epidermal regions			
Migrating cells (flattened)	–	+	++/+++
Proliferating cells *(S. basalis)*	–/+	+	++
Nascent *S. spinosum*	++	–	+
Nascent *S. granulosum*	+++	–	–
Hypertrophic (adjacent to wound)	+++	–	–
Hair follicle (outgrowths)	–	–	++
Hair follicle (different layers)	+/+++	–	–
Sweat ducts		–	
Secretory glands		+	
Mesenchymal regions			
Eschar (dead tissue)	–	–	–
Granulation tissue (wound bed)	+		+
Inflammatory infiltrate (pericapillary)	+	++	++
Endothelial cells	–	+/–	–/+++
Arrectory pili muscle		–	
Smooth muscle/artery	+/–	+	+
Neutrophils			
Macrophages	+++	+++	+++
Fibroblasts			

(–) Staining not detectable; (+) faint staining; (++) moderate staining; (+++) intense staining.
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layer provides residual epidermal protection so that when wounding or infection occur, this ligand can be released and bind receptor on leukocytes, keratinocytes, and endothelial cells to facilitate wound repair and/or inhibit infection.

3.1.6. Other Wound-Healing Models

CXCR2 is also reported to be expressed in the brain and spinal cord tissues where it may be involved in mediating neuronal function *(117)*. It is expressed in the hippocampus, dentate nucleus, pontine nuclei, locus coeruleus, paraventricular nucleus, and in the anterior horn, intermediolateral cell column and Clarke's column of the spinal cord. Patients with Alzheimer's disease expressed CXCR2 in the neuritic portion of plaques surrounding amyloid deposits, suggesting a potential role for this receptor in neurodegenerative disorders *(117)*. Another chemokine receptor, the DARC receptor, is expressed in the central nervous system, excusively in the Purkinje cells of the cerebellum. Transgenic mice expressing KC, the murine homolog of MGSA and IL-8 under the transcriptional regulation of myelin basic protein, targeting expression to neuronal cells, exhibit a neurological syndrome involving failure to maintain postural stability and rigidity beginning at 40 d of age. Mice from one transgenic line frequently died prior to one year of age and exhibited blood–brain barrier disruption that did not involve dysmyelination and extensive activation of the microglial cells was observed *(118)*. These investigators postulated that both neutrophil recruitment into the CNS and direct effects of KC on neuronal cells might contribute to the phenotype of these animals.

3.1.7. Effects of CXC Chemokines on Endothelial Cells and Angiogenesis

MGSA/GRO, like IL-8, is angiogenic in both the in vitro endothelial chemotaxis assay and in the in vivo corneal neovascularization assay *(61)*. MGSA/GROα is equivalent to IL-8, whereas MGSA/GROβ is less angiogenic *(18)*. This angiogenic activity requires the presence of a conserved N-terminal ELR motif *(18)*. IP-10 and antibodies to MGSA/GROα will block a large percentage of the angiogenic response from squamous-cell carcinoma *(61)*. Strieter has postulated that in normal tissues there is a balance between the ELR containing CXC chemokines that are angiogenic and the non-ELR-containing CXC chemokines that are antagonists of angiogenesis *(18,119)*. During tumorigenesis, this balance is shifted such that the ELR containing CXC chemokines predominate and hasten tumor formation *(36)*.

Shono et al. have verified that IL-8 enhances tubular morphogenesis in microvascular endothelial cells and that antibody to IL-8 will block that response *(120)*. In contrast, a recent study by Cao et al. in Folkman's laboratory suggests that the MGSA/GROβ protein inhibits angiogenesis when introduced at microgram concentrations and has no effect on angiogenesis at lower concentrations *(121)*. Continuous exposure to microgram concentrations of chemokine might be expected to cause downregulation and desensitization of receptors for the chemokine *(122)*. At present, it is unclear why those investigators failed to observe a chemotactic response with lower concentrations of ligand. Recently, it has been demonstrated that culturing endothelial cells on the appropriate collagen matrix is required to preserve the expression of the MGSA/GRO receptor on these endothelial cells normally observed in vivo at sites of neovascularization *(123)*. We have documented the expression of the MGSA/GRO receptor, CXCR2, on endothelial cells undergoing neovascularization in human burn wounds and in melanoma tumors *(105,124)*. In collaboration with Burdick and Strieter *(65)*, we also have demonstrated

that MGSA/GRO proteins produced by melanocytes expressing the MGSA/GROα, β, γ transgenes are angiogenic in the rabbit cornea micropocket assay and that antibodies specific for these MGSA/GRO proteins will block that angiogenic response *(65)*. These data demonstrate that chemokines can stimulate an angiogenic response that can be specifically inhibited with blocking antisera to MGSA/GRO. Thus activation of a chemokine receptor/binding protein by either MGSA/GRO, IL-8, or other CXC chemokines is a major mode of tumor-induced angiogenesis that provides nutrients for the growing tumor. However, other receptors or chemokine binding moieties such as proteoglycans expressed in endothelial cells could also participate in regulating this angiogenic response. One potential candidate is DARC, though as yet this receptor has not been shown to transduce an intracellular signal.

4. Cloning and Characterization of a New Mouse Receptor That Has 75% Homology to the Human DARC

It is not clear what mouse CXC chemokine receptor is responsible for the angiogenesis associated with MGSA/GRO-mediated wound healing or tumorigenesis. The expression of the mouse homolog of CXCR2 has been interrupted by targeted gene knock-out experiments and there were no defects in angiogenesis or organogenesis *(42)*. Therefore we sought to clone other mouse CXC chemokine receptors that might be involved in angiogenesis in response to chemokines. In humans DARC is expressed predominantely on endothelial cells and red blood cells. Using the human DARC receptor cDNA probe, a mouse genomic library was screened and mDARC full-length clones were isolated and sequenced. We determined that this genomic clone contains an intron 5' of the coding sequence *(125)*. We have mapped this gene to mouse chromosome 1 between Xmv41 and D1Mit166. Northern blot analysis revealed the pattern of receptor expression is similar to that of human tissues with expression in heart, brain, spleen, lung, liver, and skeletal muscle. Brain and skeletal muscle contained a much larger mRNA that hybridized with the mDARC probe. Similar observations have been made when human brain RNA was probed for human DARC. When the expression of this mouse DARC was examined by Northern blot analysis of mouse embryos between days 8–17 after meals, we found that expression was quite strong between 9.5 and 14 d, after which it declined. This is a time during which there is extensive organogenesis accompanied by expansion of the circulatory system. Another group has also cloned mDARC and confirmed earlier reports on the ligand-binding profile of this receptor showing that MCP-1, RANTES, IL-8, eotaxin, and MGSA/GRO α and β compete with [^{125}I]-MGSA/GRO for binding sites on murine erythrocytes and transfected K562 cells *(124a)*. Human DARC binds MGSA/GRO, IL-8 as well as the CC chemokines, MCP-1 and RANTES.

We have probed a number of rat hemangiosarcoma lesions that developed in spleens of Eker rats with loss of function of the tumor suppressor, Tsc-2, which leads to development of renal-cell carcinoma, hemangiosarcoma, and uterine leiomyoloma. Based on Northern analysis, we find that in four of five of these hemangiosarcomas, there is a very high expression of rat DARC *(125)*. The one tumor that expressed lower levels of rat DARC expressed higher levels of the rat homolog of the CXCR2. DARC is thought to serve as a ligand "sink" possibly to remove excess chemokine after injury or inflammatory response, though alternate functions have been proposed. These data indicate that this mouse receptor homolog may be very useful in examining the role of the DARC in angiogenesis during wound healing.

5. Melanoma

5.1. MGSA/GRO Ligand and Receptor Are Expressed in Both Nevus and Melanoma Tissues

In the Hs294T melanoma cell line that was the source of the first isolation of MGSA protein and the cloning of the MGSA/GRO cDNA, endogenous expression of MGSA/GRO mRNA was quite high in the absence of exogenous cytokine stimulation *(23)*. Recent studies from my laboratory show that this endogenous expression of MGSA/GRO is the result of high basal transcription of the MGSA/GRO gene *(23)*. Transcription of MGSA/GRO is dependent upon the cis-acting elements NF-κB, HMG(I)Y, Sp1, and an immediate upstream regulatory element, the IUR *(23,124,126,127)*. In Hs294T melanoma cells turnover of IκB is much more rapid than in control retinal pigment epithelial cells and this results in an endogenous nuclear activation of NF-κB and endogenous transcription of MGSA/GRO *(128)*. Melanoma tumor immunohistochemistry data confirm the presence of MGSA/GRO protein in melanoma tumors and some benign nevi *(129–131,148)*, as well as the presence of infiltrating lymphocytes and inflammatory infiltrates in melanoma *(132–135)*. After surveying a large number of primary cultures of metastatic melanoma lesions for MGSA/GRO production, we found that greater than 70% of the lesional cultures contained cells that produced MGSA/GRO protein, whereas in contrast most of the cells in the nevus cultures did not produce detectable levels of MGSA/GRO protein *(130,136)*. We have also recently shown that CXCR2 is expressed in 7/11 tumors studied and that in general, as IP-10 levels decline, MGSA/GRO expression increases *(124)* (Table 4). In contrast, mRNA isolated from cultured normal melanocytes contain little mRNA for MGSA/GRO *(130,137,138)*. More recently, a study by Herlyn's group at the Wistar demonstrated that MGSA/GROα mRNA was expressed in 100% of the melanoma lesions (21/21) based upon RT-PCR technique *(139)*. Cultured human melanocytes (5/5) also revealed MGSA/GROα mRNA by RT-PCR in the Herlyn study *(139)*, but not by Northern analysis in our study *(130)*, indicating that the levels of the mRNA are very low in melanocytes. Based upon recent studies on the role of translation in the regulation of mRNA degradation we would suspect that the untranslated mRNA in the nevi and melanocyte cultures is more stable and therefore detectable in these cultures. However, presence of the mRNA in these instances does not appear to correlate positively with MGSA protein expression/secretion *(140)*.

Schadendorf et al. have also shown that IL-8 produced by human malignant melanoma cells in vitro is an essential autocrine growth factor *(141,142)*. Antibodies to IL-8 protein and IL-8 antisense oligonucleotides inhibited the growth of two malignant melanoma cell lines in soft agar (SK-MEL-13 and SK-MEL 23). Six of the eight melanoma cell lines examined expressed detectable levels of IL-8 by RT-PCR *(142)*. This role of CXC chemokines in melanoma was emphasized further by the work of Moser et al. *(143)* with the demonstration that melanoma cells express the IL-8R mRNA in 2 melanocyte and 19 melanoma cell lines tested. Using RT-PCR we have shown that the mRNA for CXCR1 and CXCR2 are present in cultured melanoma cells *(124,144)*. Metzner et al. have confirmed the presence of CXCR2 on these cells using antibodies to the N terminus of this receptor and they have also demonstrated that MGSA/GRO α induced calcium transients in Fura-2-labeled melanoma cells *(145)*. Thus, the expression of mRNA for MGSA/GROα as well as IL-8 and the receptors for these ligands on melanocytes and melanoma cells appears to be a frequent event. The expression of ligand protein, however, is much higher in melanoma than in normal cultured melanocytes and

there is little evidence that the CXC chemokine or chemokine receptor mRNAs are translated in normal melanocytes not stimulated by cytokines such as IL-1 or TNFα *(129,130,146,147)*. Finally, Horuk's laboratory as well as work by Roby and Page have suggested that melanoma cells might make a novel receptor for MGSA/GRO *(58)*. However, additional verification and characterization of these novel receptors is needed.

5.2. ELR⁺ CXC Chemokines Also Affect Melanoma Cell Migration and Metastasis

Wang et al. demonstrated that IL-8 is haptotactic for melanoma cells in vitro, possibly contributing to the secondary localization of tumors at sites of inflammation *(149)*. To follow this up, Singh et al. *(150)* recently demonstrated that highly metastatic melanoma cell lines produce higher levels of IL-8 than melanoma cell lines with low metastatic potential and addition of IL-8 to those melanoma cell lines with low metastatic potential stimulated the proliferation of those cells. Singh et al. have followed up on this to demonstrate that UV-irradiation of a culture established from a primary melanoma that did not express IL-8 and was neither tumorigenic nor metastatic in nude mice, led to elevations in IL-8 mRNA that coincided with onset of tumor-forming capacity and metastasis in Balb/c nude mice *(151)*. It was postulated that IL-8 might act through an autocrine mechanism to affect melanoma cells and through a paracrine mechanism to enhance angiogenesis. By inducing the 72-kDa collagenase this chemokine can also impact metastatic potential.

MGSA/GRO is mitogenic for normal melanocytes, nevocytes, and melanoma cells. As a single agent and in combination with TPA and either IGF-1 or insulin, it stimulates as well as the combination of bFGF, TPA, and insulin *(130)*. Expression of MGSA/GRO or IL-8 in melanocytes is associated with enhanced growth, ability to form tumors in nude mice, and enhanced metastatic capacity in melanoma tumors *(63,65,142,150,151)*. We have demonstrated that antibodies to MGSA/GRO *(152)* and its receptor (A. Richmond, unpublished), block >50% the ligand binding and growth of the Hs294T cells. Norgauer *(153)* recently demonstrated using Fab fragments of a blocking CXCR2 antibody that this receptor is expressed on five malignant melanoma cell lines at levels 7- to 1.3-fold greater than the Hs294T cells. The level of expression for normal melanocytes was only 50% of that of Hs294T and 15-fold less than for the A2058 line. The secretion of MGSA/GRO in these cell lines over 24 h ranged from 2060 pg to 784 as compared to 126 pg for melanocytes. Hayashi et al. have demonstrated that the hexapeptide, antileukinate, which inhibits the binding of MGSA/GRO to its receptor, will completely suppress the growth of Hs294T and RPMI7951 melanoma cell lines at concentrations of 100 μ*M* peptide and this growth inhibition can be reversed by addition of excess MGSA/GRO *(154)*.

We have shown that transformation occurs following overexpression of recombinant human MGSA/GROα, β, γ in the murine melanocyte cell line, Melan-a, by transfecting melanocytes with plasmid DNA containing the MGSA/GRO cDNA placed under the control of the cytomegalovirus promoter/enhancer *(65)*. Indication of the transformed phenotype included formation of colonies in soft agar and tumors in nude mice. Tumors developed in approx 100% of the mice injected with MGSA/GRO expressing Mel-a-6 cells (Tables 5 and 6). In contrast, a clone expressing a low level of MGSA/GRO (Mel-a-1) and control transfectants expressing the neo-vector alone each yielded tumors in only 1 of 9 mice. The melanoma tumors from the mice injected with Mel-a-6 cells expressed reduced S-100 protein and increased levels of the melanoma specific antigen

Table 4
Immunohistochemistry Detection of the Expression
of MGSA/GROα, CXC2, and IP-10 in Human Melanoma

Case	Antibody	Tumor Cells	Endothelial Cells	Macrophages	Other
1. MM	MGSA/GROα	Some ++	—	—	—
Lymph node	CXCR2	—	—	—	—
	IP-10	—	—	—	CT±
2. MM	MGSA/GR0α	—	—	—	—
Lymph node	CXCR2	++	—	—	F +
	IP-10	—	Some +	—	—
3. MM/liver	MGSAα	—	—	—	—
	CXCR2	—	—	—	—
	IP-10	—	—	+	CT ±
4. MM	MGSA/GROα	Some ++	—	—	—
Lymph node	CXCR2	Some ++	—	+	—
	IP-10	+	—	—	—
5. MM	MGSA/GROα	Some +	Some +	—	—
Lymph node	CXCR2	Some ±	—	—	—
	IP-10	Some ±	—	—	—
6. Secondary	MGSA/GROα	+++	—	++	F++
cutaneous	CXCR2	Some ±	Some +	+	F+
melanoma	IP-10	Some ±	—	—	—

7. MM	MGSA/GROα	++	—	+	F++
Breast	CXCR2	—	—	—	—
	IP-10	—	—	—	—
8. Primary	MGSA/GR0α	++	++	++	E ++++
melanoma	CXCR2	+++	+++	—	E ++++
(nevus)	IP-10	—	—	—	—
9. MM	MGSA/GROα	++++	++	++	L ++
Lymph node	CXCR2	±	—	—	L +
	IP-10	+	±	—	CT ++
10. MM	MGSA/GROα	—	+++	++	F +++
Leg	CXCR2	Some ±	—	—	—
	IP-10	+	—	—	F + +
11. MM	MGSA/GROα	—	+++	++	F +
Leg	CXCR2	±	—	—	SM +
	IP-10	±	–	+	CT ±

Paraffin-embedded tissues were processed and immunostained with specific antisera as previously described.

(±) slightly positive; (+) positive; (++) moderately positive; (+++) strongly positive; (++++) very strongly positive; (CT) connective tissue; (E) epidermis; (F) fibroblasts; (L) lymphocytes; (MM) malignant melanoma; (SM) smooth muscle.

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Table 5
Tumor Formation and Angiogenic Responses
for MGSA/GRO Expressing Melanocytes

MGSA-expressing cell line	Tumor formation	Nude mice cornea angiogenesis responses		
		With condition medium	With NRS	With chemokine antibody
Mel-a-6	6/6	6/6	5/6	0/8
γ3-14	7/9	6/7	5/6	1/5
γ3-12	9/9	N.D.[a]	N.D.	N.D.
γ1-37	9/9	N.D.	N.D.	N.D.
β2-19	9/9	4/6	5/6	1/6
β2-5	9/9	3/6	N.D.	N.D.
β2–13	9/9	N.D.	N.D.	N.D.
V-1	0/15	0/5	N.D.	N.D.
V-4	0/6	N.D.	N.D.	N.D.
V-6	2/6	2/6	N.D.	N.D.
Hs294T	5/5	N.D.	N.D.	N.D.

The MGSA/GROα-expressing clone (Mel-a-6), MGSA/GROβ expressing clones (β2-5, b2-13, b2-19), MGSA/GROγ-expressing clones (γ3-12, g3–14, γ1-37), and vector control clones (V–1,V–4, V–6) were injected into nude mice. Tumor formation results are shown in the table. Hydron pellets of serum-free culture medium concentrated from mel-a-6, β2-19, γ3-14, V–1, and V–4 were implanted into the rat cornea as previously described *(17)*. Angiogenic responses were shown by MGSA/GRO-expressing clones 6 d later, and angiogenic responses were inhibited by antibodies to respective MGSA/GRO protein, but not by normal rabbit serum (NRS).

[a] N.D. = Not Determined.

HMB-45. We recultured the neomycin-resistant cells from the tumors that formed, reinjected them into nude mice, and saw the tumors develop again, even more rapidly than the first time. Culture medium from these tumorigenic melan-a cells expressing MGSA/GRO, but not from melanocytes expressing the neomycin resistance vector alone, are highly angiogenic in the rat cornea model, and antibodies to the expressed human chemokine (MGSA/GROα, β, γ) will block that angiogenic response (Table 5). These data demonstrate that MGSA/GRO is also a paracrine mediator of tumorigenesis *(18,64)*. Moreover, we have recently demonstrated that antibodies to MGSA/GROα slowed melanoma tumor growth in SCID mice and antibody to MGSA/GROγ almost totally blocked tumor growth *(36)* (Table 6). These data indirectly implicate a role for CXCR2 in tumorigenesis and the angiogenesis associated with tumor growth. In a recent screen of human melanoma tumors, CXCR2 expression was observed in tumor cells within the lesion in approx 70% of the cases. However, only one melanoma tumor exhibited strong expression of CXCR2 in the the tumor cells. Regarding the ratio of MGSA/GRO to IP-10, in general, the tumor cells exhibited higher expression of MGSA/GRO than IP-10 *(36)*.

5.3. Other Tumor Models in which MGSA/GRO May Be Involved

Transgenic rats carrying the *HTLV-IpX* gene under the control of the H-2Kd promoter develop mammary carcinoma in the females starting at approx 5 mo of age *(162)*. The tumors exhibited massive granulocytic infiltration and there was also systemic granulocytosis and hepatosplenomegaly. CINC and MIP-2 were highly expressed in the tumor

Table 6
Inhibition of Melanoma Tumor
Growth/Angiogenesis by Antibody to MGSA/GRO

MGSA-expressing positive cell Cell-line antibody	Tumor formation		SCID mice mean tumor volume (cm^3)	
	With NRS	With antibody	With NRS	With antibody
Mel-a-6	6/6	5/6	1.1	0.7
γ_3-14	6/6	3/6	0.6	0.1
V-1	0/6	–	–	–
V-4	0/6	–	–	–

The MGSA/GROα expressing clone (Mel-a-6), MGSA/GROγ expression clone (γ_3-14), and vector control clones (V–1, V–4) were injected into SCID mice. Every other day these mice received an injection of 500 μL of antibodies to the respective MGSA/GRO protein at the site of melanocytes injection, and control groups received the same course of injection with 500 μL of normal rabbit serum (NRS). Tumor growth was followed by ruler measurement of palpatable tumors. At the time of sacrifice, total tumor volume was measured by water displacement. The sample size was six for γ_3-14 with NRS, one for γ_3-14 with antibody treatment, five for Mel-a-6 NRS and antibody treatment groups. The standard deviation for the γ_3-14 with the NRS group was 14.6, for the Mel-a-6 with NRS was 3.6, and for Mel-a-6 with antibody was 12.1. SCID = severe combined immunodeficiency disease.

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tissues. Moreover, mammary carcinoma cell lines have been shown to exhibit chemotaxis in response to MGSA/GRO as well as other chemokines, suggesting a potential role for MGSA/GRO in mammary tumor cell migration, invasion, and metastasis *(156)*.

6. Summary

In summary, present data support the concept that MGSA/GRO plays a role as a normal modulator of growth and wound healing in several cell types including melanoblasts, nevocytes, keratinocytes, synovial fibroblasts of rheumatoid arthritis patients, lymphocytes, and monocytes. It recruits neutrophils, stimulates release of granules containing enzymes from polymorphonuclear leukocytes, and weakly stimulates the oxidative burst in neutrophils. It also affects the migration of T-lymphocytes, vascular endothelial cells, vascular smooth muscle cells, basophils, and monocytes *(64,157–161)*. These interactions appear to be mediated through the binding of MGSA/GRO to CXCR2, a receptor shared with IL-8, and several other CXC chemokines. ELR motif containing CXC chemokines moderate cell growth in concert with other growth substances. Overexpression of the *MGSA/GRO* gene is associated with the transformed phenotype of a number of tumor cell lines. Though MGSA/GRO is very prevalent in normal keratinocytes *(60)*, under normal circumstances these MGSA/GRO mRNA levels appear to be tightly regulated in melanocytes, with little if any MGSA/GRO mRNA being detected by Northern blot analysis. When immortalized melanocytes continuously expressed MGSA/GRO, the cells developed the ability to form tumors in nude mice. These data suggest that although MGSA/GRO is a normal component of keratinocytes,

continuous expression in melanocytes is associated with tumor formation. It is not yet clear which receptor mediates the effects of MGSA/GRO on melanocytes. It is also possible that the tumorigenic effects of MGSA/GRO are enhanced by an angiogenic effect of this chemokine.

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