

# The tumorigenic and angiogenic effects of MGSA/GRO proteins in melanoma

Hamid Haghnegahdar,\* Jianguo Du,\* DingZhi Wang,\* Robert M. Strieter,<sup>§</sup> Marie D. Burdick,<sup>§</sup> Lillian B. Nanney,\*<sup>††</sup> Nancy Cardwell,<sup>†</sup> Jing Luan,\* Rebecca Shattuck-Brandt,\* and Ann Richmond\*<sup>‡</sup>

\*Departments of Cell Biology and <sup>†</sup>Plastic Surgery, Vanderbilt University School of Medicine; <sup>‡</sup>Department of Veterans Affairs, Nashville, Tennessee; and <sup>§</sup>Department of Internal Medicine, University of Michigan, Ann Arbor

**Abstract:** Continuous expression of the MGSA/GRO $\alpha$ ,  $\beta$ , or  $\gamma$  chemokine bestows tumor-forming capacity to the immortalized murine melanocyte cell line, melan-a. The mechanism for this transformation is unclear, although both autocrine and paracrine processes are possible because melan-a cells as well as endothelial cells express a low level of the receptor for this ligand. To further define the role of MGSA/GRO proteins in melanocyte transformation, two types of experiments were designed to neutralize the biological effects of MGSA/GRO in the transfected melan-a clones: (1) the effect of neutralizing antiserum to MGSA/GRO proteins on melan-a tumor growth was assessed; (2) the tumor-forming capacity of melan-a clones expressing ELR motif-mutated forms of MGSA/GRO with compromised receptor affinity was compared to the tumor-forming capacity of clones expressing wild-type MGSA/GRO. These experiments revealed that SCID mice inoculated with MGSA/GRO $\alpha$ - or  $\gamma$ -expressing melan-a cells and subsequently treated with antiserum to the respective chemokine exhibited decreased tumor growth. This reduction in tumor growth was accompanied by declining angiogenic activity in MGSA/GRO $\gamma$ -expressing tumors. Moreover, athymic nude mice injected with melan-a cells expressing ELR-mutant forms of MGSA/GRO $\alpha$  exhibited markedly impaired tumor-forming capacity compared with those mice injected with melan-a clones expressing wild-type MGSA/GRO. These data suggest that continuous expression of MGSA/GRO proteins may facilitate tumor growth by stimulating the growth of microvessels into the tumor (paracrine) and by affecting melanocyte growth (autocrine). *J. Leukoc. Biol.* 67: 53–62; 2000.

**Key Words:** chemokines · angiogenesis

## INTRODUCTION

CXC chemokines encoding the ELR motif (ELR<sup>+</sup>) at the amino terminus have been demonstrated to exhibit angiogenic activity in a variety of contexts [1–7]. In contrast, those CXC chemokines not containing this motif (ELR<sup>-</sup>) are angiostatic [4, 8, 9].

Angiogenic activity exhibited by ELR<sup>+</sup> CXC chemokines can be inhibited with antibodies to these chemokines [1, 3, 5, 10–14]. The angiogenic response to ELR<sup>+</sup> CXC chemokines interleukin-8 (IL-8), MGSA/GRO, or ENA-78 appears to be mediated through the binding of these ligands to CXC chemokine receptors [15, 16]. The expression of receptors for ELR<sup>+</sup> chemokines on endothelial cells in wounded tissues and in both endothelial cells and tumor cells in melanoma and head and neck squamous cell carcinoma (HNSCC) tumors has been verified [15–17].

We have previously demonstrated that overexpression of MGSA/GRO proteins in immortalized melanocytes is associated with enhanced ability to form large colonies in soft agar and enhanced ability to form tumors in nude mice [18, 19]. To determine whether the tumor formation in stable clones expressing wild-type MGSA/GRO proteins was directly linked to chemokine production and not the result of random clonal selection, we designed a study in SCID mice where it is possible to follow the effects of administration of rabbit antibodies to the human MGSA/GRO proteins on tumor growth, without obtaining an antigenic response to the rabbit antiserum. Mice were inoculated with immortalized melan-a cells expressing MGSA/GRO proteins and either antiserum to MGSA/GRO or normal rabbit serum (control) was administered every-other day into the inoculation site to determine whether direct inhibition of the chemokine would alter tumor growth. In the SCID mouse model antiserum to MGSA/GRO $\gamma$  almost totally blocked tumor formation by MGSA/GRO $\gamma$ -expressing melan-a cells, whereas antiserum to MGSA/GRO $\alpha$  only partially retarded the tumor formation by melan-a cells expressing MGSA/GRO $\alpha$ . We were better able to demonstrate a direct role for MGSA/GRO $\alpha$  in tumor growth by following tumor formation of melan-a clones expressing MGSA/GRO $\alpha$  mutated in the ELR motif, a conserved motif required for high-affinity binding to the receptor. In this study we demonstrate that melan-a cells expressing MGSA/GRO $\alpha$  mutated in the ELR motif exhibit markedly compromised ability to form tumors. To determine whether the MGSA/GRO antibody treatment of SCID mice reduced tumor growth by blocking the growth of new blood

Correspondence: Ann Richmond, Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232. E-mail: ann.richmond@mcm.vanderbilt.edu

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vessels into the tumor, we monitored the expression of the endothelial cell surface marker, CD31, in frozen sections of tumors from SCID mice. In mice where tumors developed, we observed that reduced tumor size correlated with reduced CD31 immunoreactivity. These data suggest that MGSA/GRO proteins affect tumor growth through both autocrine and paracrine mechanisms.

## METHODS

### Design of expression vector for MGSA/GRO proteins

A portion (550 bp) of the MGSA/GRO $\alpha$  cDNA including the entire open reading frame was subcloned into pALTER and mutagenesis was performed according to standard protocols to produce MGSA/GRO $\alpha$  with the following mutations: E6A, L7A, R8A, and ELR/AAA. Mutated sequences including this entire open reading frame were subsequently subcloned into the pRC/CMV expression vector and plasmid DNA was transfected into immortalized melan-a cells that were the kind gift of Dortha Bennett. Stably transfected clones were selected for neomycin resistance and maintained in selective medium, as previously described [18]. Levels of mutated MGSA/GRO released into the culture medium were quantitated by enzyme-linked immunosorbent assay (ELISA) with the use of the Quantikine kit from R & D Systems. Conditioned medium was collected from confluent cultures after 48 h of conditioning in medium containing serum. The level of expression of mutant MGSA/GRO was equivalent to that of the melan-a clone expressing wild-type (WT) MGSA/GRO $\alpha$  (WT, 0.78 ng/mL; E6A, 0.89 ng/mL; L7A, 0.70 ng/mL; R8A, 1.01 ng/mL; AAA, 1.11 ng/mL).

### Tumorigenesis models

Melan-a clones selected to express MGSA/GRO $\alpha$ , MGSA/GRO $\alpha$  with mutations in the ELR motif (E6A, L7A, R8A, and ELR/AAA), or the neomycin-selectable marker alone ( $V_1$ ) were injected into female nude mice ( $5 \times 10^6$  cells per mouse) as previously described with an  $n$  of 10 mice per cell type [18]. Tumor formation was monitored over a period of 8 months.

Melan-a clones previously selected to express MGSA/GRO $\alpha$ ,  $\beta$ ,  $\gamma$  or the neomycin selection marker alone [19] were injected into SCID mice. Cells ( $5 \times 10^6$ ) from clones  $\gamma$  3–14 expressing MGSA/GRO $\gamma$ , mel-a-6 expressing MGSA/GRO $\alpha$ , or  $V_1$  or  $V_4$  clones expressing the neomycin selection marker alone, were injected subcutaneously into the interscapular region of 6-week-old female SCID mice, with an  $n$  of 12 mice per clone type. At the time of inoculation of the MGSA/GRO $\alpha$  or MGSA/GRO $\gamma$  expressing melan-a clones, mice were also injected with decomplexed neutralizing antiserum to MGSA/GRO $\alpha$  or MGSA/GRO $\gamma$ , respectively, or NRS. For each group, six of the mice were injected with 500  $\mu$ L of NRS and the other six mice were injected with 500  $\mu$ L of antiserum to MGSA/GRO $\alpha$  or  $\gamma$ , depending on the form of MGSA/GRO expressed by the transfected clone. Injections were continued every-other day over the course of several weeks, until tumor size reached an approximate diameter of 1–1.5 cm. Mice receiving melan-a cells expressing the neomycin selection marker alone (vector controls) did not receive injections of serum or antiserum. When palpable tumors developed, the tumor volume was estimated by measuring the diameter of the tumors on a weekly basis. When any of the tumors in the group reached a diameter of 2.5 cm<sup>3</sup>, all the mice in the group were killed, the tumors were resected, and the tumor volumes were measured by water displacement. Tumors were then divided into aliquots for (1) fixation, embedding, sectioning, and histological evaluation; (2) ELISA determination of chemokine expression; and (3) analysis of frozen sections for CD31 immunodetection of endothelial cells.

### Western blot analysis of Ras protein in MGSA/GRO-expressing melan-a clones

Western blots were performed on lysates of MGSA/GRO $\alpha$ ,  $\beta$ ,  $\gamma$  expressing melan-a clones to detect the expression of Ras proteins using protocols developed by Santa Cruz. The pan-Ras antibody that detects H-Ras, K-Ras,

and N-Ras (Oncogene Research Products) was used at a 1:3000 dilution in TBS-T buffer with 5% dry milk for 12–16 h at 4°C. The membrane was then incubated in a 1:3000 dilution of the anti-mouse antibody conjugated with horseradish peroxidase (Boehringer Mannheim Corp.) in TBS-T buffer with 5% dry milk for 1 h at room temperature. The protein bands were detected with the enhanced chemiluminescence system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The density of the bands on the developed blot was quantitated and plotted. The blot was performed three times with similar results each time.

### AP-1 transient transfection assays

AP-1 luciferase reporter gene constructs were prepared by inserting two copies of the AP-1 consensus DNA binding sequence into the pGL-3 promoter vector (Promega). Semiconfluent cultures ( $5 \times 10^5$  cells in 30-mm dishes) of mel-a cells expressing MGSA/GRO $\alpha$ ,  $\beta$ , or  $\gamma$  were transiently co-transfected with 2.5  $\mu$ g pSV- $\beta$ -Gal control vector (Promega) and 2.5  $\mu$ g AP-1 luciferase reporter gene with or without 5  $\mu$ g of a dominant-negative Ras (DN-Ras) expression construct using the calcium phosphate technique. Three hours later, the cells were glycerol shocked for 3 min. The cells were washed twice with complete medium, and then incubated with complete medium at 37°C/5% CO<sub>2</sub>. Two days later, the cells were washed with cold phosphate-buffered saline (PBS) and lysed in 1 $\times$  reporter lysis buffer (Promega) for 15 min at room temperature, and the lysate was cleared by centrifugation. The luciferase and  $\beta$ -Gal activity were measured according to standard protocols (Promega) using a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

### Immunodetection of CD31 staining, mCXCR2, and the CD16 natural killer (NK) marker in tumor sections

Paraformaldehyde-fixed tissues were sectioned and processed for staining for mCXCR2 using antibody to mCXCR2 from Santa Cruz Biologicals (1:200 dilution). CD16 antibodies were purchased from PharMingen (1:200 dilution). Frozen sections of tumors were fixed in cold acetone and stained for CD31 using the antibody kindly provided by Alberto Montovani, which is also available through PharMingen [20]. Our standard protocols were used with this rat antibody for indirect immunohistochemical detection using the Vector Laboratories ABC Rat IgG kit. The substrate used for the peroxidase-linked avidin-biotin complex was 3-amino-9-ethylcarbazole (AEC) from Vector Laboratories (Burlingame, CA). Slides were counterstained with Mayer's hematoxylin, dehydrated, and mounted with aqueous immu-mount (Shandon Inc., Pittsburgh, PA). Quantitation was by morphometric scanning using protocols developed and published previously [21].

### Corneal micropocket/angiogenesis assay

The ability of extracts from tumors expressing chemokines to stimulate angiogenesis in the avascular cornea of F344 rat eyes was assayed according to established procedures [1, 22, 23]. Tumor samples were ground and sonicated, and then a 100- $\mu$ L aliquot was combined from each of six tumors arising in either the NRS-treated or antiserum-treated SCID mice to yield a combined sample of 600  $\mu$ L from each group. The MGSA/GRO antiserum-treated group of tumors had only two tumors for analysis so 300  $\mu$ L from each of these tumors was combined to produce the 600- $\mu$ L sample. Specimens were concentrated by Speed-Vac, reconstituted in 50  $\mu$ L of sterile water, and total protein was measured. Samples were adjusted for equal amounts of total protein, and pellets were made for analysis of the pooled extract in the corneal micropocket assay.

### ELISA assay

Levels of immunoreactive MGSA/GRO $\alpha$  and  $\gamma$  were determined using a modified double-ligand ELISA according to procedures previously described [22, 24]. ELISA assays were also performed for human MGSA/GRO  $\alpha$  and  $\gamma$ , murine macrophage inflammatory protein-2 (MIP-2), murine KC, murine interferon- $\gamma$  (IFN- $\gamma$ ), and murine IL-12 and IL-13 with the R & D Systems Quantikine assay. Aliquots of conditioned medium (50  $\mu$ L) from cultured melan-a cells or aliquots of tumor tissue sonicated in lysis buffer were analyzed in 96-well microtiter plates coated with rabbit anti-chemokine antibodies according to our established procedures. Data from tumors are reported in

picograms per milligram protein. Protein determinations were by the Bio-Rad protein assay kit.

## MGSA/GRO receptor expression in melan-a cells

Confluent cultures of melan-a cells expressing the neo vector alone, MGSA/GRO $\alpha$  (mel-a-6), MGSA/GRO $\beta$  ( $\beta$ 2-19), and MGSA/GRO $\gamma$  ( $\gamma$ 3-14) were assayed for  $^{125}\text{I}$ -MGSA $\alpha$  binding as previously described. Nonspecific binding was monitored by determining the binding of radiolabeled ligand in the presence of 100-fold excess cold ligand. Receptor expression was also monitored by immunocytochemistry on cultured melan-a clones expressing the various forms of MGSA/GRO and by immunohistochemistry of tissue sections of tumors that arose in nude or SCID mice. The Santa Cruz antibody to CXCR2 was used as the primary antibody in combination with the Vectastain ABC immunostaining protocols and kits.

## RESULTS

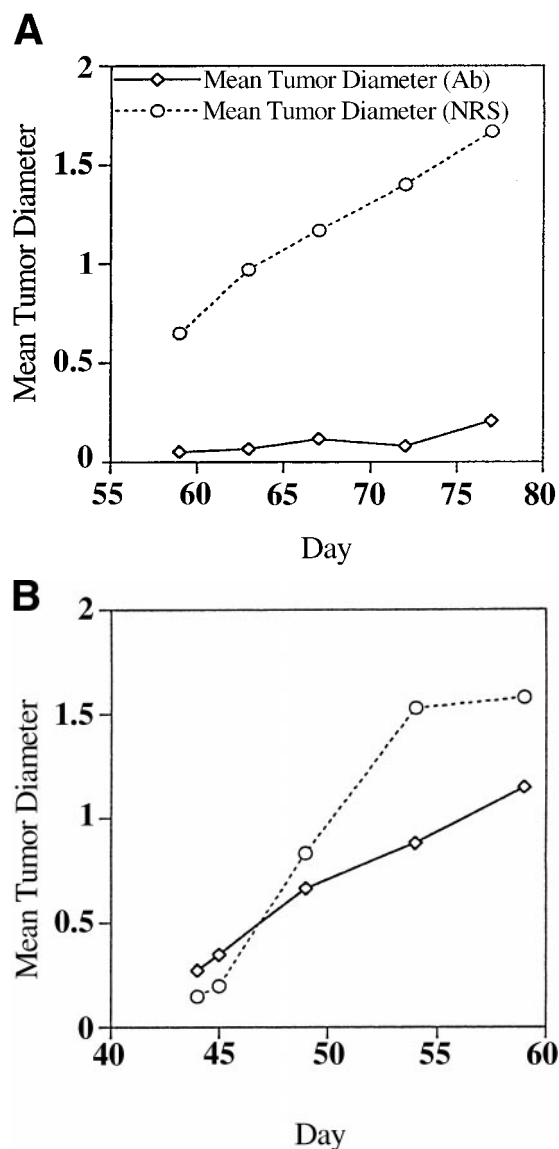
### Effect of antiserum to MGSA/GRO on tumor growth of MGSA/GRO $\alpha$ - or $\gamma$ -expressing clones

Control SCID mice inoculated with melan-a clones expressing MGSA/GRO $\alpha$  or  $\gamma$  and injected with NRS developed palpable tumors between 44 and 59 days after injection of cells. MGSA/GRO $\gamma$ -expressing tumors grew rapidly from a mean tumor diameter of 0.65 cm on day 59 to 1.67 cm by day 77 (tumor volume measurement by fluid displacement, 0.62 cm<sup>3</sup>). In contrast, only three mice in the group injected in the same manner with antiserum to MGSA/GRO $\gamma$  developed tumors and those three tumors remained very small, with a mean volume for the three tumors of 0.15 cm<sup>3</sup> (0.04 cm<sup>3</sup> by micrometer calculation) and a mean diameter of 0.1 cm (Fig. 1). Tumor sizes were significantly different ( $P < 0.05$ ) using a two-sample  $t$  test assuming unequal variances. When antiserum injections were stopped, these SCID mice developed tumors that grew rapidly, with a growth rate comparable to that of the mice receiving normal rabbit serum (NRS) injections (data not shown).

Mice injected with melan-a cells producing MGSA/GRO $\alpha$  developed palpable tumors by day 45. All six of the mice receiving injections of NRS every-other day developed tumors and these tumors grew to a mean tumor diameter of 1.58 cm, or a tumor volume as measured by fluid displacement of 1.1 cm<sup>3</sup> by 59 days. For the SCID mice inoculated with MGSA/GRO $\alpha$ -expressing melan-a cells and injected with antiserum to MGSA/GRO $\alpha$ , five of the six mice developed tumors and these tumors grew more slowly than in the mice injected every-other day with NRS. The antibody-treated tumors reached a mean diameter of only 1.1 cm, or a volume of 0.75 cm<sup>3</sup> based on fluid displacement (1.05 cm<sup>3</sup> by micrometer calculation), by 59 days after the initial inoculation into the mice (Fig. 1). The  $P$  value for differences in mean tumor size between NRS and antibody treatment for MGSA/GRO $\alpha$ -expressing tumors did not achieve statistical significance ( $P = 0.103$ ).

### Effect of mutation of the ELR motif on MGSA/GRO induction of tumor formation

Because the growth of MGSA/GRO $\alpha$ -expressing tumors was not fully inhibited by antibody to this chemokine, a second strategy was developed to neutralize the activity of this ligand. We



**Fig. 1.** Effect of antiserum to MGSA/GRO $\alpha$  or MGSA/GRO $\gamma$  on the growth of melan-a tumors in SCID mice. Melan-a cells ( $5 \times 10^6$ ) expressing MGSA/GRO $\gamma$  (A) or MGSA/GRO $\alpha$  (B) were injected into 12 young female SCID mice. (A) Mice from each group (expressing MGSA/GRO $\alpha$  or  $\gamma$ ) were injected every-other day with de complemented blocking antiserum to these respective chemokines or with heat-inactivated normal rabbit serum. We compared the mean tumor diameter as determined by micrometer measurement in the group injected with antibodies (Ab) to MGSA/GRO or to that of the group injected with normal rabbit serum (NRS). Three mice of the six treated with the antiserum to the MGSA/GRO $\gamma$  slowly developed very small tumors (over 2.5 months; final mean tumor diameter ranged from 0.7 to 0.2 cm for the three out of six mice that developed tumors), whereas those six mice treated with NRS quickly developed very large tumors by 8 weeks after the initial injection of melan-a cells expressing MGSA/GRO $\alpha$  (final tumor diameters ranged from 2.3 to 1 cm). (B) For 8.5 weeks antiserum to MGSA/GRO $\alpha$  (0.5 mL) or NRS (0.5 mL) was injected every-other day into SCID mice inoculated with MGSA/GRO $\alpha$ -expressing melan-a cells (clone mel-a-6). The diameter of the tumors was monitored weekly by micrometer measurement. The MGSA/GRO $\alpha$ -expressing cells made tumors in five out of six mice injected with antibody to MGSA/GRO $\alpha$  (final tumor diameters ranged from 2.0 to 0.8 cm for the five out of six mice developing tumors) while tumors arose in all six of the mice injected with NRS (final tumor diameters ranged from 2.5 to 1.0 cm).

produced ELR motif mutations in MGSA/GRO $\alpha$  to compromise the high-affinity binding of this chemokine to its receptor, CXCR2. Stable melan-a clones expressing four different mutations in the ELR motif were established: E6A, L7A, R8A, and ELR/AAA. Mice injected with melan-a cells expressing mutant (ELR<sup>-</sup>) ELR-MGSA/GRO $\alpha$  exhibited a marked reduction in tumor formation compared with those expressing wild-type (ELR<sup>+</sup>) MGSA/GRO $\alpha$  (Table 1). No tumors formed in the nude mice injected with the E6A mutant clone. Approximately 5.5 months after injection, only 2–4 of the 10 mice injected with cells expressing the L7A or R8A mutations, respectively, formed tumors. Much later (7.5–8 months after cell inoculation), small nodules (2–10 mm) had begun to form on mice injected with the clones expressing these ELR<sup>-</sup> mutant forms of MGSA/GRO $\alpha$ . In general, the tumors that formed in mice expressing ELR motif mutant forms of MGSA/GRO $\alpha$  did not develop until 5–8 months after injection of cells, whereas mice injected with melan-a cells expressing wild-type MGSA/GRO $\alpha$  formed tumors within 2.5 months after injection (see Table 1). The incidence of tumor formation for the melan-a cells expressing ELR/AAA mutant MGSA/GRO $\alpha$  was similar to that of melan-a cells expressing the neomycin-selectable marker alone (V<sub>1</sub>). These data directly link the requirement for MGSA/GRO $\alpha$  activation of its receptor through an intact ELR motif with induction of tumorigenesis.

#### Receptor expression in melan-a cells and in tumors arising in mice injected with MGSA/GRO-expressing melan-a cells

Ligand binding assays on melan-a cells expressing MGSA/GRO $\alpha$ ,  $\beta$ ,  $\gamma$ , or neo vector controls using <sup>125</sup>I-MGSA revealed comparable specific binding for V<sub>1</sub> control, MGSA/GRO $\alpha$ ,  $\beta$ , and  $\gamma$  expressing clones (1477, 1575, 1717, 1369 cpm, respectively). Specific binding was determined in triplicate after subtracting the counts per minute bound in the presence of 100-fold excess cold ligand. We estimate that this represents approximately 10,000 binding sites per cell.

Immunostaining of the cells in the growing tumor revealed that a subpopulation of cells within the tumor stained positively with antibody to CXCR2 (Fig. 2, A–C). However, the majority of the cells within the tumors did not exhibit mCXCR2 immunoreactivity. Moreover, blood vessels within the tumor reacted strongly with mCXCR2 antibody, suggesting that the secreted MGSA/GRO available from the melan-a cells could cause these endothelial cells to induce an angiogenic response (Fig. 2D). These data suggest that as the tumor forms there may be both autocrine and paracrine roles for MGSA/GRO stimulation of tumor growth.

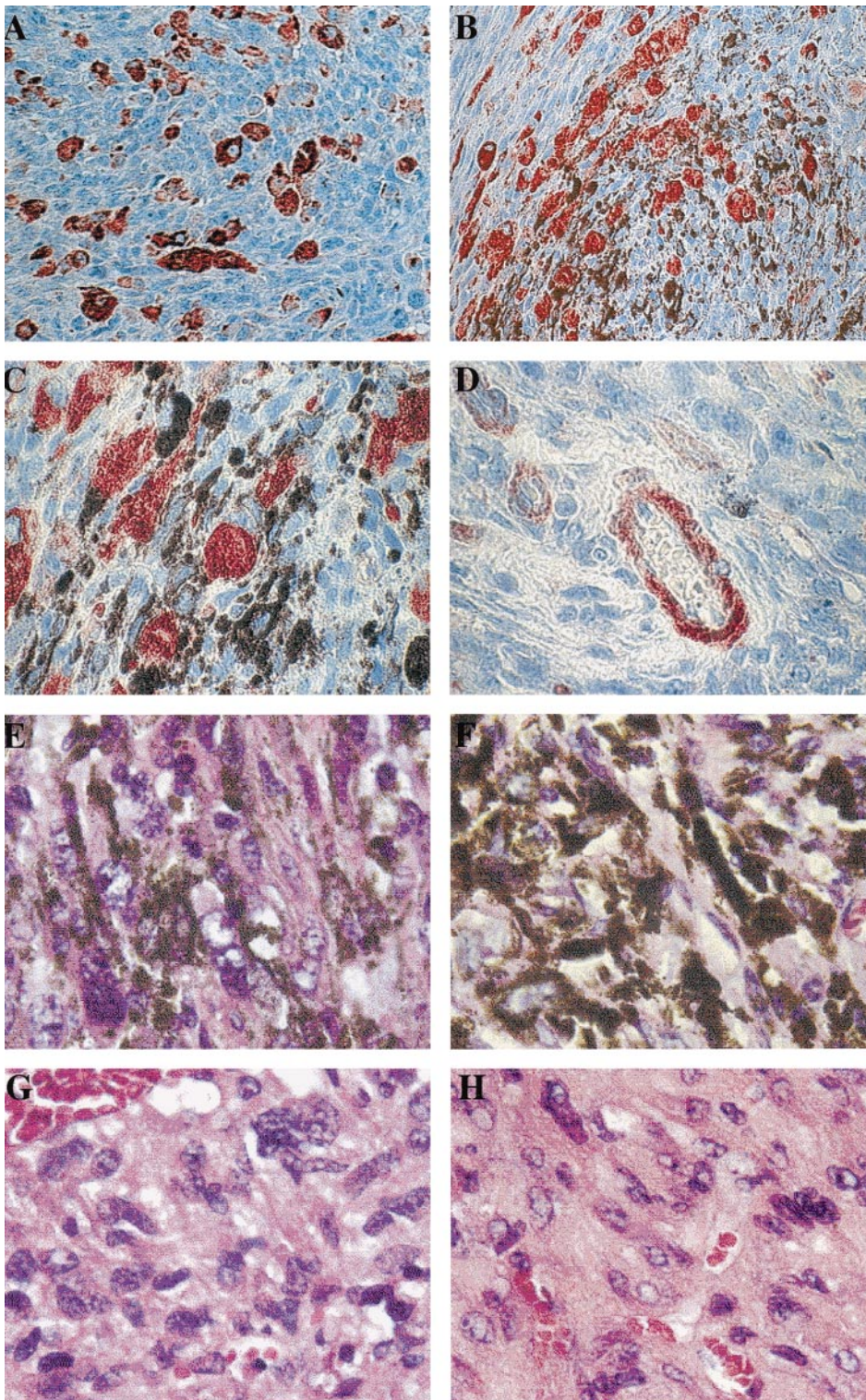
#### Histology of tumors and detection of inflammatory infiltrate or NK cells

To determine whether the antibody treatment of the tumors resulted in binding of antibody to NK cells through the Fc receptor, and potential activation of these cells to enhance cell killing, we examined the histology of the H & E-stained tumors and attempted to localize NK cells within the tumors through the use of specific markers for NK cells (CD16). Histological examination of the tumors that arose in the MGSA/GRO $\alpha$  or  $\gamma$

TABLE 1. Effect of Mutation of the ELR Motif of the MGSA/GRO Proteins on Tumor Formation

	Tumor latency period	Total period of tumor growth	Volume of tumor at removal	Diameter of small tumors
<b>MUTANT L7A</b>				
Mouse 1	3.5	5 months	1.0 ml	
Mouse 2	5.5	7.5 months	2.5 ml	
Mouse 3	5.5	8 months	3.5 ml	
Mouse 4	5.5	8 months	3.0 ml	
Mouse 5	6.5			10 mm
Mouse 6	7.5			6–7 mm
Mouse 7	8			4–5 mm
Mouse 8	8.5			2–3 mm
Mouse 9	8.5			2–3 mm
Mouse 10	8.5			2–3 mm
<b>MUTANT R8A</b>				
Mouse 1	4.5	6 months	1.3 ml	
Mouse 2	5	7.5 months	2.5 ml	
Mouse 3	5.5	8 months	2.0 ml	
Mouse 4	7.5			6–8 mm
Mouse 5	7.5			6–8 mm
Mouse 6	7.5			6–8 mm
Mouse 7	7.5			6–8 mm
Mouse 8	No tumor formed			
Mouse 9	No tumor formed			
Mouse 10	No tumor formed			
<b>MUTANT AAA</b>				
Mouse 1	5.5	7.5 months	3.0 ml	
Mouse 2	5.5	7.5 months	2.5 ml	
Mouse 3	8.5			2–3 mm
Mouse 4	No tumor formed			
Mouse 5	No tumor formed			
Mouse 6	No tumor formed			
Mouse 7	No tumor formed			
Mouse 8	No tumor formed			
Mouse 9	No tumor formed			
Mouse 10	No tumor formed			
<b>WT MGSA</b>				
Mouse 1	1.5	2.5 months	5.0 ml	
Mouse 2	1.5	2.5 months	2.5 ml	
Mouse 3	1.5	2.5 months	2.0 ml	
Mouse 4	2	3 months	2.5 ml	
Mouse 5	2	3 months	2.5 ml	
Mouse 6	2	3 months	2.0 ml	
Mouse 7	2	3 months	2.2 ml	
Mouse 8	2.5	3 months	1.5 ml	
Mouse 9				8 mm
Mouse 10				4 mm
Mouse 11				5 mm
Mouse 12				2–3 mm
E6A Mutant and V <sub>1</sub> Control	No tumors formed			

Melan-a cells were transfected with MGSA/GRO $\alpha$  expression vectors exhibiting the following mutations in the ELR motif: E/A, L/A, R/A, or ELR/AAA. Stable clones were selected based on neomycin resistance, expanded, expression of mutant protein was verified by ELISA assay, and  $5 \times 10^6$  cells were injected from each clone into female nude mice of approximately 8 weeks of age.



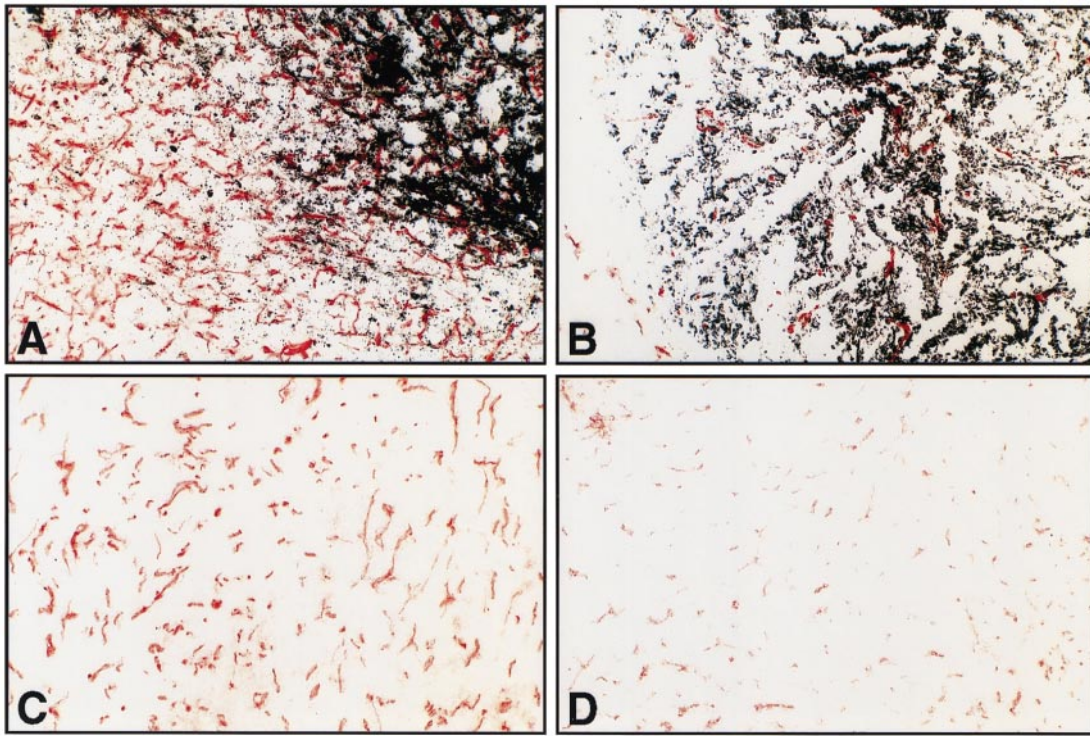
**Fig. 2.** Immunolocalization of mCXCR2 and histology of the melan-a tumors. Tumors that arose in SCID mice injected with melan-a cells expressing MGSA/GRO $\gamma$  were fixed in paraformaldehyde, embedded in paraffin, sectioned, and stained with antibody to mCXCR2 (Santa Cruz Biotechnology, Inc.) using the ABC Elite kit from Vector Laboratories, with a substrate of aminoethyl carbazole for the peroxidase, which produces a red staining indicative of the presence of the antigen, mCXCR2. (A) Note the presence of a subset of non-melanotic tumor cells that stain strongly for immunoreactive mCXCR2 (original magnification,  $\times 200$ ). (B) The presence of immunoreactive mCXCR2 tumor cells interspersed among highly pigmented tumor cells is shown (original magnification  $\times 200$ ). (C) At higher power (original magnification  $\times 1000$ ) the presence of CXCR2 immunoreactive spindle-shaped tumor cells in the area of a number of highly pigmented tumor cells is shown. (D) The presence of immunoreactive mCXCR2 in the endothelial cells of a blood vessel in the tumor that formed after injection of melan-a cells expressing MGSA/GRO $\gamma$  (original magnification  $\times 1000$ ). Additional capillaries in cross section also exhibit immunoreactivity for the mCXCR2. (E, F) The H & E staining of the MGSA/GRO $\gamma$ -expressing tumors treated with NRS (E) or antiserum to MGSA/GRO $\gamma$  is shown (F) (original magnification  $\times 1000$ ). (G, H) The H & E staining of the MGSA/GRO $\alpha$ -expressing tumors treated with NRS (G) or antiserum to MGSA/GRO $\alpha$  is shown (H) (original magnification  $\times 1000$ ). Note the typical lack of leukocytic infiltration in these tumors.

antibody-treated group or the NRS-treated group did not reveal the presence of inflammatory infiltrate or NK cells within the tumors (Fig. 2, E–H).

### Tumor vascularity assessment

To determine whether antiserum inhibition of tumor growth was due in part to inhibition of angiogenesis, we examined the vascularity of antiserum-treated tumors with the use of an immunohistochemical marker for endothelial cells, CD31 [25] (Fig. 3). Although there are visual differences in the extent of

vascularity as assessed by CD31 immunostaining, quantitation of the total immunoreactivity using 15 high-magnification microscope fields revealed little difference in the CD31 immunodetection in the antibody-treated group of tumors compared with the NRS-treated tumors (Table 2). The MGSA/GRO $\gamma$  tumors treated with NRS had a mean staining volume over the tumor of  $2208 \pm 1506$ , whereas the antiserum-treated group from the two small tumors that arose from the six mice treated gave a mean staining volume of 1345 (range 979–1710) over the tumors ( $P = 0.28$ ). Similarly, for the tumors expressing



**Fig. 3.** Immunohistochemical staining of tumor-associated blood vessels as visualized by CD31 antisera. (A, B) Heavily pigmented MGSA/GRO tumors: A, received treatment with normal rabbit serum and shows robust in-growth of blood vessels; B, received treatment with MGSA/GRO antisera and shows scant numbers of blood vessels. (C, D) Non-pigmented MGSA/GRO tumors: panel C shows moderate neovascularization after treatment with normal rabbit serum; panel D shows little evidence of neovascularization after treatment with MGSA/GRO antisera.

MGSA/GRO $\alpha$ , the mean CD31 staining volume over the tumors was  $4462 \pm 955$  from the NRS-treated serum compared with  $6168 \pm 1361$  ( $P = 0.05$ ) for the group of tumors arising in the MGSA/GRO $\alpha$  antiserum-treated group. Although the raw data indicated that the fields examined from the MGSA/GRO $\gamma$  antiserum-treated group had highly similar areas of CD31 staining, closer inspection by multiplying the total tumor volume by the area of CD31-positive vessels within tumors revealed a trend toward reduced vascularity per tumor in the antiserum-treated group compared with the NRS treatment group (vascularity index: NRS-treated MGSA/GRO $\gamma$  tumors,  $1542 \pm 1558$ ; antibody-treated MGSA/GRO $\gamma$  tumors,  $183 \pm 12$ ,  $P = 0.08$ ). A difference was also observed in the vascularity index for the MGSA/GRO $\alpha$ -expressing tumors: NRS-treated tumors,  $6016 \pm 4827$ ; anti-serum-treated tumors,  $3922 \pm 4360$ . However, differences in these means did not show statistical significance based on the two-sample  $t$  test. For tumors that arose from mel-a cells expressing both MGSA/GRO $\alpha$  and MGSA/GRO $\gamma$ , there were no significant differences in the distribution of the blood vessels with NRS or chemokine antiserum treatment.

#### Angiogenic response to tumor extracts from mice treated with NRS versus antiserum to MGSA/GRO

When extracts of the tumor lysates were analyzed for angiogenic activity in the corneal micropocket/angiogenesis assay, we observed that the MGSA/GRO $\alpha$  and MGSA/GRO $\gamma$  tumors arising in the NRS-treated group were all able to produce a

strong angiogenic response, 4/4 positive for the MGSA/GRO $\alpha$  tumor extracts and 3/3 positive for the MGSA/GRO $\gamma$  tumors (**Table 3**). In contrast, the tumors arising in the antiserum-treated animals did not yield a positive angiogenic response (3/3 negative for the MGSA/GRO $\gamma$  tumors and 4/4 negative for the MGSA/GRO $\alpha$ -expressing tumors). Thus, the antibody treatment neutralized the tumor angiogenesis.

#### ELISA determination of ELR<sup>+</sup> versus ELR<sup>-</sup> chemokines: quantitation of the levels of MGSA/GRO proteins expressed in the tumors that formed

To determine whether the growth of the MGSA/GRO $\alpha$ - and MGSA/GRO $\gamma$ -expressing tumors was differentially inhibited by antiserum to the respective chemokine because the  $\alpha$ -expressing tumors produced higher levels of ELR<sup>+</sup> chemokine, we allowed tumors to develop in SCID mice without antiserum injections, removed the tumors at the same time, then monitored the tumor levels of MGSA/GRO $\alpha$ , MGSA/GRO $\gamma$ , KC, and MIP-2 by ELISA. The results from this experiment revealed considerable variation in the level of expression of chemokine from tumor to tumor (**Table 4**). The MGSA/GRO $\alpha$  tumors produced a mean MGSA/GRO $\alpha$  of 432 pg/mg protein (range 20–1120); the MGSA/GRO $\gamma$  tumors produced a mean of only 30 pg of MGSA/GRO/mg of protein (range 9–116;  $P = 0.001$ ). There was a trend toward higher expression of KC and MIP-2 in the MGSA/GRO $\alpha$ -expressing tumors, compared with MGSA/GRO $\gamma$ -expressing tumors: MGSA/GRO $\gamma$  tumor MIP-2 levels were 45 pg/mg protein (range 4–210); KC levels were 20 pg/mg

TABLE 2. CD31 Immunostaining of Tumors from SCID Mice Treated with NRS or Antiserum to MGSA/GRO

	Relative CD31 staining units				
	Tumor	Fat	Total dermis	Tumor volume	Staining × Tumor Vol
<b>MGSA/GRO<math>\gamma</math> tumors with NRS</b>					
1	4222	6465	10,687	1.0	4222
2	3633	1984	7823	0.3	1089
3	1670	4285	5955	0.3	501
4	2312	2117	4229	1.1	2543
5	230	1396	1628	0.3	69
6	1186	1987	3173	0.7	830
Mean	2208 ± 1506				1542 ± 1558
<b>MGSA/GRO<math>\gamma</math> Tumors with Ab</b>					
1	979	3335	4314	0.2	196
2	1710	1569	3279	~0.1	171
Mean	1345				183
<b>MGSA/GRO<math>\alpha</math> tumors with NRS</b>					
1	5218	2154	7372	2.5	13045
2	2768	2114	4882	0.1	277
3	5160	2542	7702	0.7	3612
4	5126	1096	6222	1.5	9333
5	4483	984	5467	1.4	7654
6	4019	1416	5435	0.4	2174
Mean	4462 ± 955				6016 ± 4827
<b>MGSA/GRO<math>\alpha</math> tumors with AB</b>					
1	5456	2805	8261	1.0	5456
2	4955	2050	7005	2.2	10901
3	8480	1836	10,316	~.1	848
4	5908	2649	8557	~.1	591
5	6043	2248	8291	0.3	1812
Mean	6168 ± 1361				3922 ± 4360

Tumor vascularity was assessed by staining endothelial cells in acetone-fixed frozen sections of tumor with rat antibody to CD31 followed by Vector Laboratories ABC kit. Morphometric scanning was used for quantitation of the total immunoreactivity over 15 fields of tumor or fat and dermis. The staining index was determined by multiplying the relative CD31 staining over the tumor by the tumor volume, as determined by fluid displacement of the dissected tumor.

protein (range 3–117); for MGSA/GRO $\alpha$  tumors the MIP-2 levels were 197 pg/mg protein (range 24–321), and KC levels were 59 pg/mg protein (range 9–148). It has been demonstrated previously that MGSA/GRO can augment the expression of its own mRNA in cultured endothelial cells [34]. We postulate that expression of MGSA/GRO by the injected melan-a cells can also activate expression of KC and MIP-2 in the endothelial

TABLE 3. Angiogenic Activity in Tumor Lysates From SCID Mice Treated With NRS or MGSA/GRO Antiserum

MGSA/GRO $\gamma$ tumors with NRS	3/3 eyes positive
MGSA/GRO $\gamma$ tumors with MGSA/GRO antibody	3/3 eyes negative
MGSA/GRO $\alpha$ tumors with NRS	4/4 eyes positive
MGSA/GRO $\alpha$ tumors with MGSA/GRO antibody	4/4 eyes negative

Tumors from antiserum or NRS-treated SCID mice were dissected, sectioned, ground, and sonicated in buffer using previously published protocols [22, 23]. One hundred-microliter aliquots were combined to produce a sample of 600  $\mu$ L from each group, samples were concentrated and reconstituted in 50  $\mu$ L of sterile water, total protein was measured, and the hydron pellets were made for analysis through the use of the corneal micropocket assay according to standard procedures. Six rat eyes were studied from the MGSA/GRO $\gamma$  tumors and eight were studied from the MGSA/GRO $\alpha$  treatment group.

cells of the tumors and thus enhance chemokine levels. When the total mean expression of MGSA/GRO, MIP-2, and KC are totaled for the  $\alpha$ - and  $\gamma$ -expressing tumors from the experiment using five mice to calculate the mean chemokine expression, there is a trend toward higher expression of these CXC chemokines in the MGSA/GRO $\alpha$ -expressing tumors compared with the MGSA/GRO $\gamma$ -expressing tumors, although the ranges of expression overlap between these two groups: MGSA/GRO $\alpha$ -expressing clone, 95 pg/mg protein; MGSA/GRO $\gamma$ -expressing clone, 688 pg/mg protein,  $P < 0.001$  (Table 3). A similar trend was observed using the R & D Quantikine ELISA kit. Because MGSA/GRO $\alpha$ , MGSA/GRO $\gamma$ , MIP-2, and KC all bind and activate the same receptor, these total differences in ligands for CXCR2 may have a significant biological impact on the growth of tumors in the mice.

To determine whether the differences in the tumor growth and antibody inhibition between the MGSA/GRO $\alpha$  and  $\gamma$ -expressing clones were due to variation in the production of cytokines that might inhibit tumor growth or alter the recruitment of NK cells or other leukocytes, we performed ELISA assays for key cytokines on conditioned medium from these clones. The levels of IFN- $\gamma$ , IL-12, and IL-13 production by the MGSA/GRO $\alpha$ ,  $\gamma$ , and vector control melan-a clones were examined. There was no detectable release of these cytokines into the culture medium in any of the three melan-a clones (data not shown). Thus differences in tumor growth between MGSA/GRO $\alpha$ - or  $\gamma$ -expressing clones are not due to differential production of these cytokines.

#### Differential Ras expression and AP-1 activity in MGSA/GRO $\alpha$ -expressing Mel-a-6 cells compared with melan-a clones expressing MGSA/GRO $\beta$ or $\gamma$

We postulated that the differences in tumor growth of the MGSA/GRO $\alpha$ -expressing clones could be the result of the ability of MGSA/GRO $\alpha$  acting through CXCR2 on the melan-a cells to induce a secondary transforming event, which would not be sensitive to inhibition by antibody to MGSA/GRO $\alpha$ . Because ras is often involved in melanoma tumorigenesis, we examined the expression of Ras proteins in melan-a clones expressing the various forms of MGSA/GRO compared with vector controls using Western blot analysis as described in Materials and Methods. We observed that the MGSA/GRO $\alpha$ -expressing clone gave a stronger expression of Ras protein, using an antibody that would detect H, N, and K-Ras (Table 5). This enhanced expression of Ras protein in the MGSA/GRO $\alpha$ -expressing clone was associated with increased AP-1 transcriptional activity using an assay that involved transient transfection with an AP-1-luciferase reporter construct, using co-transfection with  $\beta$ -Gal to normalize for transfection efficiency (Table 5). We examined AP-1 activity in three separate clones expressing MGSA/GRO $\alpha$ , three clones expressing MGSA/GRO $\beta$ , and three clones expressing MGSA/GRO $\gamma$  (data not shown). Consistently, the MGSA/GRO $\alpha$ -expressing clones exhibited elevated AP-1 transactivation compared with the MGSA/GRO $\beta$ - and  $\gamma$ -expressing clones. These data argue that the AP-1 activation occurs in response to MGSA/GRO $\alpha$  expression and is not the result of clonal variation. Moreover, transfection

TABLE 4. Quantitation of MGSA/GRO, KC, and MIP-2 Levels in Tumors that Arose on the SCID Mice Inoculated with MGSA/GRO $\alpha$  or MGSA/GRO $\gamma$  Expressing Melan-a Cells

Tumor line	MGSA/GRO $\alpha$ , pg/mg protein	MGSA/GRO $\gamma$ , pg/mg protein	KC, pg/mg protein	MIP-2, pg/mg protein	Total
MGSA/GRO $\gamma$		30 (9–116)	20 (3–117)	45 (4–210)	95
MGSA/GRO $\alpha$	432 (20–1120)		59 (9–148)	197 (24–321)	688

ELISA assays and protein determinations were performed on sonicated tumor tissue using procedures previously described [22, 24]. Tumors that formed in antibody treatment and NRS treatment groups were tested multiple times and the mean values for each treatment group were analyzed. Mean determinations are shown; ranges of the determinations are shown in parentheses.

of a dominant-negative *Ras* construct along with the AP-1 reporter demonstrated that elimination of the endogenous *Ras* activity blocked the elevation in endogenous AP-1 activity in the MGSA/GRO $\alpha$ -expressing mel-a-6 clone (Table 5). These data suggest that constitutive expression of MGSA/GRO $\alpha$  can have secondary effects on gene expression that contribute to the stronger transforming effect of MGSA/GRO $\alpha$  compared with MGSA/GRO $\beta$  or  $\gamma$ .

## DISCUSSION

The role of CXC chemokines in angiogenesis [1, 7, 23] and wound healing [26] has been recently clarified. Squamous cell carcinoma and non-small-cell lung tumors express ELR<sup>+</sup> chemokines and the angiogenic activity in these tumors can be inhibited with antibodies to ELR<sup>+</sup> chemokines such as IL-8 [5, 11, 27, 28]. The elaboration of chemokines by tumor cells has been clearly established, and it has been postulated that release of chemokines from tumor cells expressing receptors for the ELR motif positive chemokines might also facilitate tumor growth through an autocrine mechanism [4, 5, 9, 11, 27, 29–36]. In work reported here, we demonstrate that immortalized melanocytes expressing MGSA/GRO $\alpha$  mutated in the ELR motif are compromised with regard to tumor-forming capacity. Moreover, we demonstrate that stable clones of melanocytes releasing the transgene product MGSA/GRO $\alpha$  or MGSA/GRO $\gamma$  form tumors in SCID mice and that treatment of the mice with antiserum to MGSA/GRO from the time of the initial inoculation with melanocytes expressing these chemokines results in a significant reduction in tumor growth. The murine CXCR2 receptor [37] is clearly visible on a population of the tumor cells and in the blood vessels within the tumor, being displayed prominently in the endothelial cells, pericytes, and smooth muscle cells of the vessels. Thus, it is conceivable that MGSA/GRO activation of melan-a cells and blood vessel

growth could involve CXCR2. Analysis of the ability of the tumor extracts from the antibody-treated mice revealed little angiogenic activity in these tumors in comparison to the NRS-treated mice where a full angiogenic response was observed [19]. Because the tumors showed prominent vascularity, it is apparent that some tumor-associated angiogenesis did take place during the development of the tumor, although this process had apparently been suppressed by the time the tumor was extracted and the CMA was performed. Although the ELR<sup>+</sup> murine CXC chemokines, KC and MIP-2, were present in the tumor extracts, the angiogenic potential of these ligands must have been blocked by angiostatic substances within the tumor, based on the 100% inhibition of the angiogenic response in the presence of antibodies to MGSA/GRO $\alpha$  or  $\gamma$ . A recent report from Bergers et al. shows that CD31 staining/cm<sup>3</sup> of tumor remains equivalent in tumors treated with endostatin and angiostatin, whereas tumor volume shrinks due to apoptosis of endothelial cells [39].

The antiserum to MGSA/GRO $\gamma$  almost totally blocked tumor growth *in vivo*, based on reduced tumor formation and reduced tumor volume. These antibodies also inhibited angiogenesis by 73 or 89%, based on CD31 staining. We did not observe the same quantitative antibody inhibition for clones expressing MGSA/GRO $\alpha$  compared with MGSA/GRO $\gamma$ . We postulate three possible reasons for these differences. (1) Based on the ELISA results summarized in Table 4, there may be a contribution from the somewhat higher combined level of expression of MGSA/GRO, MIP-2, and KC in the MGSA/GRO $\alpha$ -expressing cells (688 pg/mg protein) compared with the MGSA/GRO $\gamma$ -expressing cells (95 pg/mg protein). However, the *in vitro* expression of chemokines was similar for the  $\alpha$ - and  $\gamma$ -expressing cells [19]. Because the angiogenic activity of the tumor lysates was totally eliminated in the antiserum-treated tumor group (Table 3), these data suggest that the antiserum to MGSA/GRO $\alpha$  was present at sufficiently high levels to neutralize the angiogenic activity of this chemokine, even if it were

TABLE 5. Levels of *Ras* Protein and AP-1 Transactivating Activity

Clone	Vector	MGSA/GRO $\beta$	MGSA/GRO $\gamma$	MGSA/GRO $\alpha$
Ras protein expression level (fold)	1	4.15 $\pm$ 0.65	3.25 $\pm$ 0.05	7.00 $\pm$ 0.90
Basal AP-1 transactivation (fold)	1	1.12 $\pm$ 0.32	2.10 $\pm$ 0.18	3.16 $\pm$ 0.45
Dominant-negative Ras inhibits AP-1 transactivation	0.54 $\pm$ 0.10		0.48 $\pm$ 0.01	0.43 $\pm$ 0.12

The level of *Ras* protein and AP-1 transactivating activity were monitored by Western blot and by luciferase activity in the MGSA/GRO $\alpha$ -,  $\beta$ -, and  $\gamma$ -expressing melan-a clones as described in Materials and Methods. The luciferase and  $\beta$ -Gal activities were measured 48 h after transfection. Transfection efficiency was normalized by comparing AP-1 luciferase activity to  $\beta$ -galactosidase activity. The data are reported as mean  $\pm$  SEM fold induction (the relative luciferase activity of MGSA/GRO $\alpha$ ,  $\beta$ ,  $\gamma$  divided by the relative luciferase activity of the vector) from three independent experiments. In experiments involving dominant-negative Ras transfection, the results are reported as mean fold inhibition  $\pm$  SEM (the relative luciferase activity of dominant-negative form of M-Ras divided by the relative luciferase activity of pBK-CMV vector) from three independent experiments. Co-transfection of the dominant negative *Ras* expression construct with the AP-1 luciferase reporter construct resulted in a marked reduction in endogenous AP-1 reporter activity.



expressed at higher levels. Specific ELISAs for murine mig and IP-10 are not available, so we were unable to accurately access the levels of these angiostatic chemokines in the tumors that developed. (2) Alternatively, there may also be innate differences in the biological activity of MGSA/GRO $\alpha$  versus MGSA/GRO $\gamma$ . Here we show that melan-a cells expressing MGSA/GRO $\alpha$  with mutation in the ELR motif formed tumors much more slowly than melan-a cells expressing wild-type MGSA/GRO. These data demonstrate that compromising the biological activity of the MGSA/GRO $\alpha$  ligand by altering the affinity for the receptor [39] markedly reduces tumor growth. Although it has been previously demonstrated that ELR mutant forms of CXC chemokines exhibit loss of angiogenic activity [22], there has been no prior demonstration that these mutations affect tumor growth. (3) Finally, there may be quantitative differences in the cellular response to MGSA/GRO $\alpha$  compared with  $\gamma$ , which affects gene expression, and this may render the cells less responsive to antibody inhibition. We show here that there is enhanced expression of Ras protein and enhanced AP-1 transcriptional activity. These events could contribute to secondary effects on gene transcription, which could conceivably contribute to the increased transforming activity of MGSA/GRO $\alpha$  or to the partial resistance of melan-a tumors formed by clones expressing MGSA/GRO $\alpha$  to growth inhibition using antibody to the chemokine transgene product. Moreover, we have examined differences in gene expression of the  $\alpha$ - and  $\gamma$ -expressing clones using the differential display technique to determine whether other factors might be contributing to differences in the antibody inhibition of tumor growth between these two MGSA/GRO $\alpha$ - and  $\gamma$ -expressing clones. Initial results suggest that expression of MGSA/GRO $\alpha$  is a stronger inducer of expression of several genes, including *Ras*, than is MGSA/GRO $\gamma$  [Wang et al., unpublished results]. Moreover, the differences in the *Ras*-dependent constitutive AP-1 transcriptional activity in MGSA/GRO $\alpha$ -expressing clones compared with clones expressing MGSA/GRO $\beta$  or  $\gamma$  or the ELR/AAA mutant form of MGSA/GRO $\alpha$  could lead to altered transcription of other genes involved in melanocyte transformation. These differences in gene expression could also contribute to the transformed phenotype of melan-a cells and to the resistance to antiserum inhibition of growth of the MGSA/GRO $\alpha$ -expressing clones.

One potential nonspecific effect of antibody injection into SCID mice could include the activation of NK cells through the binding of the Fc portion of the antibody to the Fc receptor on the NK cells. Several lines of evidence suggest that this is not the case in the model tested here. First, we did not observe leukocytic infiltration into the developing tumors with or without antibody injection. Second, differential activation of NK cells could not explain the differences observed with the treatment of developing tumors with antibody to MGSA/GRO $\alpha$  versus MGSA/GRO $\gamma$  because we injected the same amount of antiserum into mice inoculated with cells expressing both MGSA/GRO $\alpha$  and MGSA/GRO $\gamma$ , yet the antiserum to MGSA/GRO $\gamma$  produced a greater inhibition of tumor growth.

A second potential problem with this model might be differential expression of an inhibitory substance such as IFN- $\gamma$ , IL-13, or IL-12, which could differentially affect tumor

growth in the various clones. We did not observe differences in IFN- $\gamma$  expression in the conditioned medium of these cultures (data not shown). The markedly reduced tumor burden in mice expressing ELR mutant forms of MGSA/GRO $\alpha$  also argues against the possibility that differential induction of cytokine expression by transfected bacterially produced DNA might be responsible for the differences in tumor growth observed between the  $\alpha$  and  $\gamma$  expressors. Thus, we believe that the tumor growth is occurring in response to continuous expression of chemokines.

In summary, we have shown here that continuous MGSA/GRO expression may lead to enhanced melanocyte tumor formation through autocrine stimulation of melanocyte growth, enhancement of the growth of blood vessels to the tumor, and induction of changes in gene expression. Continuous expression of the  $\alpha$ ,  $\beta$ , or  $\gamma$  forms of MGSA/GRO in melanocytes results in transformation, but the effects on melanocyte growth and gene expression may vary with the form of MGSA/GRO expressed.

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