A Synthetic Peptide Inhibitor for α-Chemokines Inhibits the Growth of Melanoma Cell Lines

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Abstract

Melanoma growth stimulatory activity (MGSA/GROα) is a 73 amino acid peptide sharing sequence characteristics with the α-chemokine superfamily. MGSA/GROα is produced by diverse melanoma cell lines and reported to act as an autocrine growth factor for the cells. We tested the binding of MGSA/GROα to melanoma cell lines, Hs 294T and RPMI-7951, and found that these cells could bind to MGSA/GROα but not to interleukin-8.

Recently, we defined a novel hexapeptide, antileukinate, which is a potent inhibitor of binding of α-chemokines to their receptors on neutrophils. When antileukinate was added to melanoma cells, it inhibited the binding of MGSA/GROα. The growth of cells from both melanoma cell lines was suppressed completely in the presence of 100 μM peptide. The cell growth inhibition was reversed by the removal of the peptide from the culture media or by the addition of the excess amount of MGSA/GROα. The viability of Hs 294T cells in the presence of 100 μM peptide was > 92%. These findings suggest that MGSA/GROα is an essential autostimulatory growth factor for melanoma cells and antileukinate inhibits their growth by preventing MGSA/GROα from binding to its receptors. (*J. Clin. Invest.* 1997. 99:2581–2587.) Key words: melanoma • neoplastic proteins • growth substances • interleukin-8 • growth inhibitor

Introduction

Melanoma growth stimulatory activity (MGSA)[1]/GROα is a peptide that was first identified as an autostimulatory growth factor of Hs 294T melanoma cells (1–3). Further studies indicated that it was produced by diverse melanoma cell lines (4) and played an important role in the tumorigenesis and growth of malignant melanoma cells (5). Sequence analysis demonstrated that MGSA/GROα is a member of the superfamily called "α-chemokines" (6). This family of proteins were chemotactic for neutrophils and had substantial sequence homology, a C-X-C motif near the amino-terminal end, and two additional Cys residues closer to the carboxyl-terminal end. The functions of α-chemokines were mediated by receptors on the cell surface membrane (7–9). Recent studies showed the presence of some types of chemokine receptors on melanoma cells. Some melanoma cells possess IL-8 receptors similar to those on neutrophils (8–11).

Recently, we defined a novel hexapeptide, antileukinate (Ac-RRWWCR-NH_2), which is a potent inhibitor of binding of α-chemokines to the receptors (12). In these studies, we examined the effect of antileukinate on MGSA/GROα binding to melanoma cell lines and found that it could suppress the growth of melanoma cells.

Methods

Peptide synthesis. A hexapeptide, RRWWCR with acetylated NH_2 terminus and amidated COOH terminus, antileukinate, was synthesized and purified by Houghten Pharmaceutical Inc. (San Diego, CA) as described previously (12). In some experiments, the antileukinate was radioactively labeled by acetylating the amino terminus with tritiated anhydrous acetic acid. This procedure was carried out at Multiple Peptide Systems (San Diego, CA). The resulting specific radioactivity was 156 Ci/mol.

Cell culture. Melanoma cell lines, Hs 294T and RPMI-7951, were purchased from American Type Culture Collection (Rockville, MD). A liver cancer cell line, Hep G2, was a gift from M.-C. Liu (University of Texas Health Center at Tyler, TX). The cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (HyClone Laboratories Inc., Logan, UT), 2 mM L-glutamine, 50 U/ml of penicillin and 50 mg/ml of streptomycin.

Binding assays. Recombinant human MGSA/GROα and IL-8 were purchased from Pepro Tech Inc. (Rocky Hill, NJ) and radioactively labeled using ^{125}I-labeled Bolton-Hunter reagent (DuPont-NEN, Wilmington, DE) (13). The labeled proteins were isolated using Sephadex G-25 column (Column PD-10; Pharmacia LKB Biotechnology, Piscataway, NJ) pre-equilibrated with PBS containing 0.1% gelatin and stored at −70°C after the addition of 1% bovine serum albumin.

Binding studies with melanoma cell lines were performed by the method of Horuk and colleagues (14). In brief, the cells (1×10^5 cells/well) in 24-well plates were washed two times with HBSS and incubated with ^{125}I-labeled MGSA/GROα or IL-8 in the buffer containing 1% bovine serum albumin at 4°C for 3 h. The incubation was terminated by vacuum aspiration of the supernatant. The cells were washed three times in binding buffer, solubilized by the addition of 200 μl of 1% SDS, and transferred to vials for counting. The nonspecific binding was estimated by measuring the binding in the presence of 100-fold excess of nonlabeled ligand. The binding parameters were calculated using the Lundon II computer program (Lundon Software Inc., Chagrin Falls, OH).

Measurement of MGSA/GROα and IL-8. MGSA/GROα in the culture supernatants was measured using an enzyme immunoassay (R & D Systems, Minneapolis, MN). IL-8 was measured with a sandwich enzyme immunoassay (15). Plates were coated with monoclonal anti–human IL-8 antibody grown and purified from hybridoma HB9647 which was a gift from Dr. E.J. Leonard (Immunopathology Section, Laboratory of Immunobiology, National Cancer Institute, Frederick,

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1. *Abbreviations used in this paper:* MGSA, melanoma growth stimulatory activity; TFA, trifluoroacetic acid.

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MD). Samples were then added and IL-8 bound to the plates was detected with a two-step incubation with rabbit polyclonal anti–human IL-8 antibody (Upstate Biotechnology Inc., Lake Placid, NY) and swine anti–rabbit immunoglobulins conjugated with horseradish peroxidase (DAKO Corp., Carpinteria, CA). Both immunoassays were specific for their antigen and did not cross-react with other members of the α-chemokine family.

Analysis of antileukinate breakdown. Hs 294T and Hep G2 cells, 2×10^4 cells/well, were cultured in 24-well tissue culture plates for 24 h. Then the culture media were replaced with media containing tritiated antileukinate, 0.2 μCi, mixed with 100 μM unlabeled peptide in each well. The cells were then cultured for the indicated periods. The supernatants were collected and stored at −70°C until use. The breakdown of antileukinate during the cell culture was analyzed on HPLC using an analytical C18 reversed phase column (Waters Co., New Bedford, MA). The peptides were eluted using a gradient from 0.1% trifluoroacetic acid (TFA) to 80% acetonitrile in 0.1% TFA. 1-ml fractions were collected and measured for their radioactivity content using a liquid scintillation counter.

Cell growth assays. The cell lines (2×10^4 cells/well) in 500 μl cell culture medium were grown in 24-well tissue culture plates. After the cells adhered to the plate, the supernatants were aspirated and 500 μl of media containing various concentrations of antileukinate were added to the wells. The cells were cultured for period indicated in the text. The culture media were changed to fresh media every 24 h. After incubation, the cells were detached with trypsin-EDTA solution (Life Technologies Inc., Gaithersburg, MD, containing 0.05% of trypsin and 0.53 mM of EDTA in HBSS without Ca^{2+} and Mg^{2+}) and the cell number was counted using a hemocytometer.

Measurement of cytotoxic activity of the peptide. The cell lines (4×10^5 cells/well) in RPMI-1640 medium containing 10% fetal bovine serum were incubated with 10 μCi of $Na_2{}^{51}CrO_4$ (DuPont NEN Co.) for 60 min at 37°C in a 24-well tissue culture plate. The cells were washed three times and then incubated in cell culture medium for 30 min at 37°C to allow spontaneous lysis of marginally viable cells. After washing twice, a 200-μl aliquot of the antileukinate in cell culture medium was added to each well. The cells were cultured for 16 h at 37°C and then the radioactivity in the supernatants was counted in a gamma radiation spectrometer. Quadruplicate wells received cell culture medium alone or 2% SDS to determine spontaneous and maximum release, respectively. The percentage lysis was calculated by using the following formula:

$$\%Lysis = \frac{(experimental\ cpm - spontaneous\ cpm)}{(maximum\ cpm - spontaneous\ cpm)} \times 100.$$

Statistics. The data are expressed as the mean and SD unless otherwise noted. Significance of differences between multiple groups was tested using the analysis of variance and Scheffe's test.

Results

The effect of antileukinate on the binding of MGSA/GROα to melanoma cells. The binding of radiolabeled MGSA/GROα to melanoma cell lines was specific and saturable (Fig. 1). The K_d values calculated were $\sim 6 \times 10^{-9}$ M and 3×10^{-9} M for Hs 294T and RPMI-7951, respectively. The maximal binding (B_{max}) of MGSA/GROα to the melanoma cell lines was 11 fmol/10^5 cells and 15 fmol/10^5 cells, respectively. Thus, the number of MGSA/GROα binding sites was estimated to be 70,000–100,000/melanoma cell. There was, however, no specific binding of IL-8 to either cell line (Fig. 2).

Antileukinate inhibited the binding of MGSA/GROα to both of the melanoma cell lines. When the melanoma cell lines, Hs 294T and RPMI-7951, were incubated with ^{125}I-labeled MGSA/GROα, 1nM, in the presence of antileukinate, the binding was inhibited by antileukinate with an EC_{50} of ~ 1 μM (Fig. 3).

The production of α-chemokines from the melanoma cell lines. The supernatants of melanoma cells cultured for 48 h were tested for the presence of α-chemokines. The concentration of MGSA/GROα was 24.3±0.8 ng/ml in Hs 294T and 2.6±0.1 ng/ml in RPMI-7951 supernatants (Table I). IL-8 was detected at much higher concentrations in the supernatants from both melanoma cell lines (Table I). Liver cancer cell line, Hep G2, also produced detectable amounts of IL-8, however, the concentration was only a small fraction of that seen in the melanoma cell cultures. MGSA/GROα could not be detected in the supernatants of Hep G2 cells.

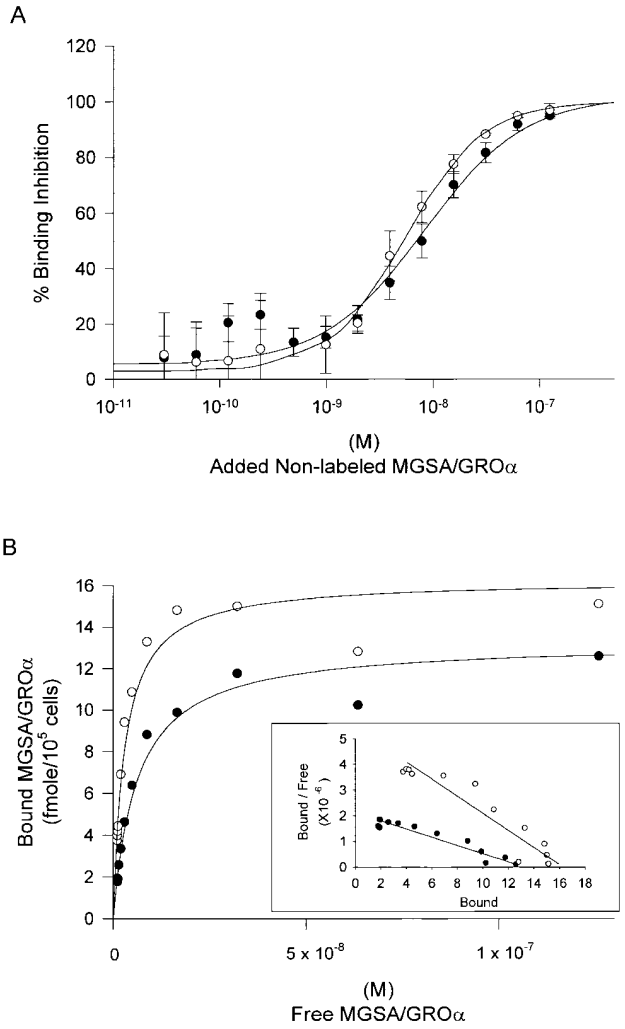

Figure 1. The binding of ^{125}I-labeled MGSA/GROα to melanoma cell lines, Hs 294T and RPMI-7951. The melanoma cells, 1×10^5, were incubated with 1 nM ^{125}I-MGSA/GROα in the presence of various concentrations of unlabeled MGSA/GROα. (*A*) Binding inhibition curve with Hs 294T (*closed circles*) and RPMI-7951 (*open circles*). The lines shown in the graph are the best fit curves calculated using the London II computer program. (*B*) Binding isotherm transformation. The total amounts of labeled and unlabeled MGSA/GROα bound to the cells were calculated. Scatchard plots were shown in the inset.

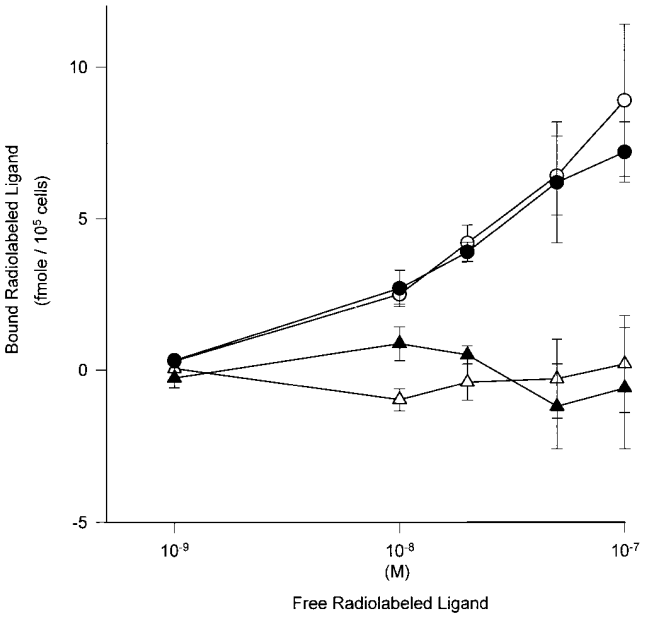

Figure 2. The specificity of the receptor binding. The melanoma cells, Hs 294T (*closed symbols*) and RPMI-7951 (*open symbols*), were incubated with indicated concentrations of radiolabeled MGSA/GROα (*circles*) or IL-8 (*triangles*). The nonspecific binding was estimated by the binding in the presence of 100-fold excess of unlabeled material. Specific binding which is calculated by subtracting non-specific binding from total binding is shown in this figure. IL-8 did not bind specifically to the melanoma cells.

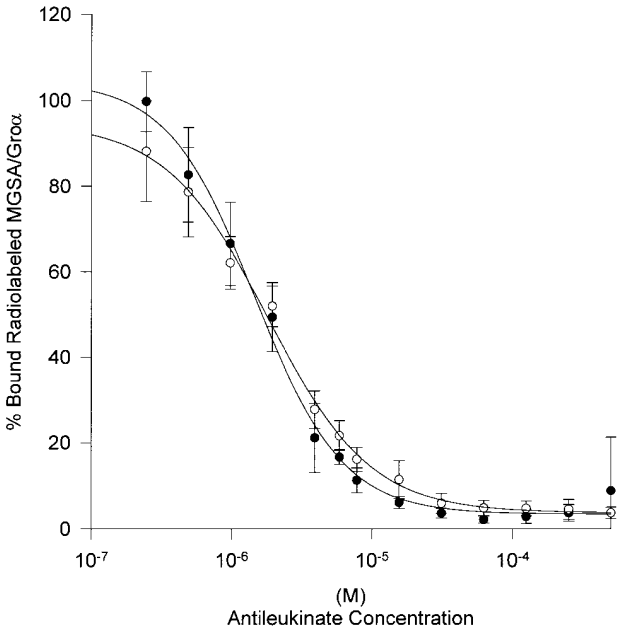

Figure 3. The effect of antileukinate on the binding of radiolabeled MGSA/GROα to the melanoma cell lines. The melanoma cells, Hs 294T (*closed circles*) and RPMI-7951 (*open circles*), were incubated with 1 nM ^{125}I-MGSA/GROα in the presence of different concentrations of antileukinate. The lines shown in the graph are the best fit curves calculated using the London II computer program.

The effect of antileukinate on the growth of melanoma cells. Since antileukinate inhibited the binding of MGSA/GROα to melanoma cell lines, we tested the ability of antileukinate to suppress autostimulatory growth of melanoma cells. Before these experiments, we examined the time dependent breakdown of antileukinate by Hs 294T melanoma cells and Hep G2 liver cancer cells. When the radiolabeled antileukinate in RPMI-1640 medium was analyzed by HPLC, the major peak of the radioactivity was eluted at 50–51 ml (Fig. 4). After a 24-h culture period, the major peak had decreased by > 85% and there was a transient increase in the peak seen at 41-42 ml (Fig. 4). These findings showed that most antileukinate in both cell cultures disappeared within 24 h by a two step catabolic process. Therefore, in later experiments we exchanged the culture medium containing antileukinate every 24 h to maintain the effect of antileukinate.

The growth of melanoma cell lines was inhibited by antileukinate. We cultured cell lines in the presence of various concentrations of antileukinate for 72 h and counted the number of cells in the well. The growth of Hs 294T cells was significantly inhibited by 10 μM peptide (Fig. 5 *A*) with an ED_{50} of 18 μM. Antileukinate also suppressed the growth of RPMI-7951 melanoma cells with an ED_{50} of 31 μM (Fig. 5 *B*); however, it did not affect the growth of Hep G2 liver cancer cells (Fig. 5 *C*).

The inhibition of Hs 294T growth was further characterized. It was reversed by the removal of antileukinate from the culture media (Fig. 6 *A*) and by the addition of 50 nM MGSA/GROα to the media (Fig. 6 *B*). These findings suggest that the

Table I. IL-8 and MGSA/GROα in the Culture Supernatants

	Origin of cell line	IL-8*	MGSA/GROα*
		ng/ml	*ng/ml*
Hs 294T	Melanoma	178.3±1.0	24.3±0.8
RPMI-7951	Melanoma	138.2±0.7	2.6±0.1
Hep G2	Liver	2.0±0.0	< 0.06

*The cells (5×10^4) were cultured in a 24-well plate for 48 h. The supernatants were collected and the concentrations of chemokines were measured as described in the text.

Table II. Cytotoxic Test of Antileukinate on Cultured Cell Lines

	Percentage cell lysis*		
Peptide added	Hs 294T	RPMI-7951	Hep G2
μM			
0	0.0±4.5	0.0±2.7	0.0±11.0
1	−4.0±5.2	−0.8±2.2	−2.1±7.5
5	−3.2±7.2	−2.6±3.7	−0.8±8.3
20	−1.6±3.7	−3.4±6.3	5.8±8.3
100	2.4±7.9	6.8±11.1	0.9±2.9

*The cells labeled with ^{51}Cr were cultured in the presence of various concentrations of antileukinate for 16 h. Then the radioactivity in the supernatants was counted. The percentage cell lysis was calculated based on the amount of chromium released from the cells during the culture.

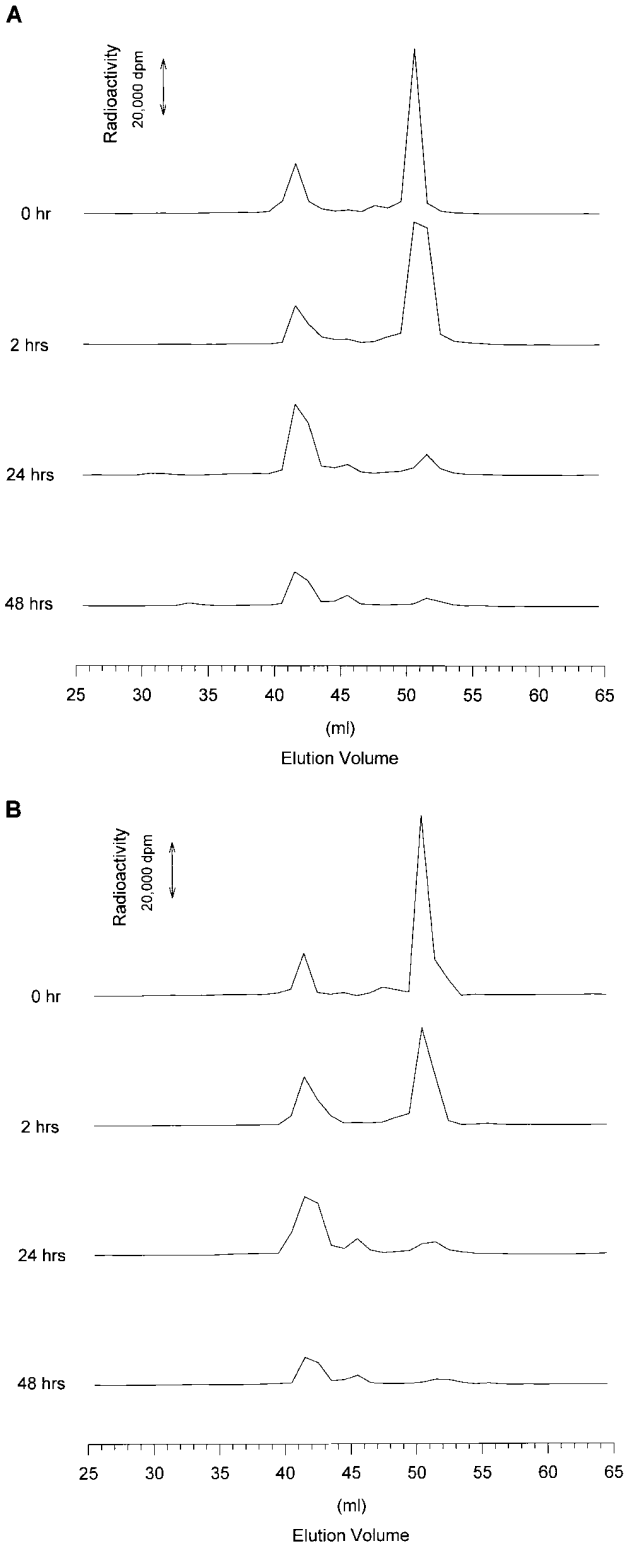

Figure 4. Analysis for the breakdown of antileukinate in the culture of Hs 294T cells and Hep G2 cells. A total concentration of tritium labeled and unlabeled antileukinate of 100 μM, was added to the culture of Hs 294T cells (*A*) and Hep G2 cells (*B*). Aliquots of medium were removed at different times and chromatographed on an HPLC Bondapak C18 reversed phase column. The peptides were eluted using a gradient from 0.1% TFA to 80% acetonitrile in 0.1% TFA. 1-ml fractions were collected and measured for their radioactivity using a liquid scintillation counter. The chromatographs show the elution volume of the radioactivity at different times after addition to the cells. The peptide is substantially eliminated from the cultures within 24 h.

Hs 294T growth inhibition by antileukinate is associated with the binding inhibition of MGSA/GROα to the receptors on the Hs 294T cell surface.

Cytotoxicity of antileukinate. To exclude the possible contribution of cytotoxicity of antileukinate to the inhibition of melanoma cell growth, we cultured ^{51}Cr-labeled cells in the presence of the peptide for 16 h. Antileukinate did not cause cell lysis of either of the melanoma cell lines or Hep G2 cell line (Table II). Measurements of cell viability by Trypan Blue exclusion also indicated that antileukinate was not toxic to the cells (Fig. 6). The adhesivity of Hs 294T, RPMI-7951 and Hep G2 cells to the culture wells did not alter when antileukinate was added (data not shown).

Discussion

MGSA/GROα is a 73 amino acid peptide which shares sequence characteristics of a superfamily of peptides called α-chemokines. Richmond and colleagues initially discovered that melanoma cells secreted autostimulatory (autocrine) growth factors (1–3, 16). They found that most of the activity was caused by a single acid stable protein of ~ 16 kD and designated it melanoma growth stimulatory activity (MGSA) (1, 2). MGSA was found to be a mitogen for the melanoma cell line Hs 294T which produces this factor. MGSA was secreted by diverse melanoma cell lines but not by benign nevus cell lines (4), while immunoreactive MGSA was shown in both types of cells (17). cDNA for MGSA isolated from Hs 294T cells was later found to be identical to oncogene growth-related peptide (GROα) gene (18). The formal name for this protein was then designated as MGSA/GROα.

Recently, a second chemokine was found to be important for melanoma cell growth and metastasis in some melanoma cell lines. Schadendorf and colleagues determined that some melanoma cell lines tested secreted IL-8 (19). Both of two IL-8 secreting cell lines studied in more detail were dependent on IL-8 for growth. Antisense oligonucleotides targeted against human IL-8 mRNA inhibited cell proliferation, colony formation in soft agar, and secretion of IL-8 into culture supernatants. In an analysis of 13 different human melanoma cell lines, it was shown that expression of IL-8 correlates with the metastatic potential of melanoma cells in BALB/c nude mice (20).

An interesting experiment further supports the importance of these chemokines in melanoma growth and tumorigenesis. Mintz and Silvers developed a method of producing melanomas by grafting skin from Tyr-SV40E transgenic mice which are highly susceptible to melanoma to Tyr-SV40E hosts of a low susceptibility of the same inbred strain (5). They suggested that growth factors and cytokines known to be produced in wound repair, may trigger the growth and malignant conversion of melanocytes. The association of some human melanomas with an earlier severe blistering sunburn may be an example of a wound-healing process initiating melanoma. This observation is particularly interesting since recent studies indicated that human skin cells express α-chemokines after injury. Nanney and colleagues showed that MGSA/GROα and its re-

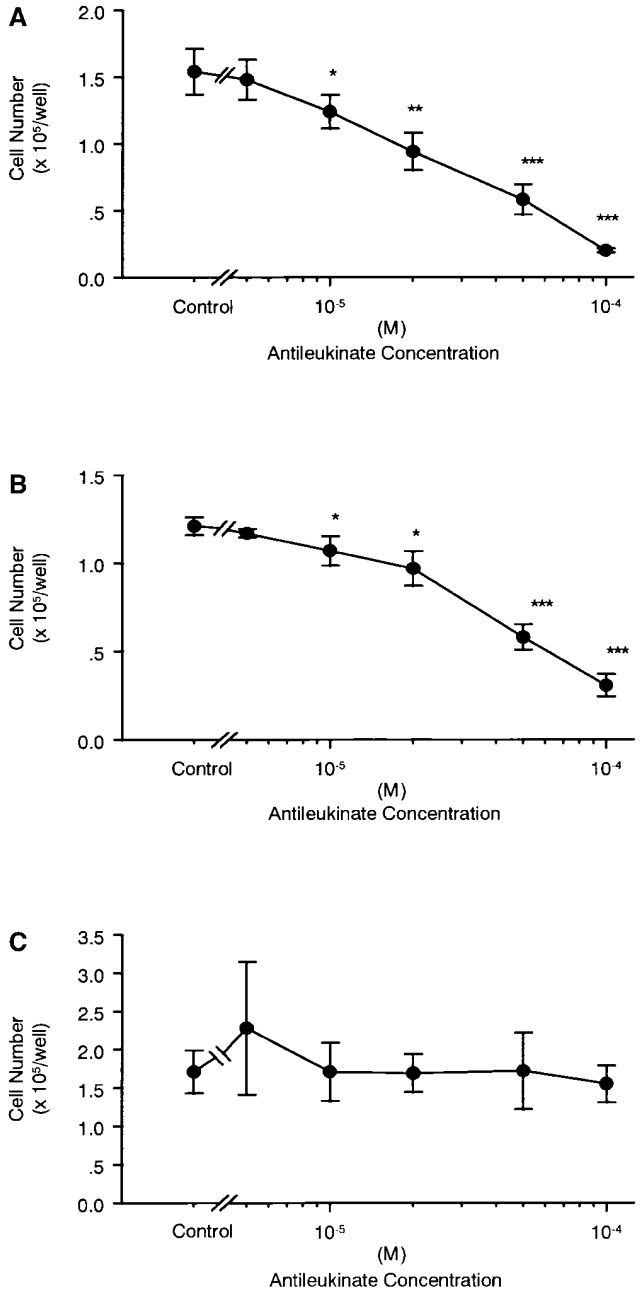

Figure 5. The effect of antileukinate on the growth of melanoma cells. Melanoma cell lines and a control liver cancer cell line (2×10^4 cells/well) in 500 μl were cultured in a 24-well tissue culture plate in the presence of various concentrations of antileukinate for 3 d. The culture media were changed to fresh media containing the same concentration of antileukinate every 24 h during the incubation period. After incubation, the cells were collected and the cell number was counted using a hemocytometer. (*A*) Hs 294T cell line. (*B*) RPMI-7951 cell line. (*C*) Liver cancer cell line, Hep G2. Analysis of variance was used for multiple comparison. When there was significant difference, the differences between the number of cells without the peptide and those with peptide were tested using the Scheffe's test; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

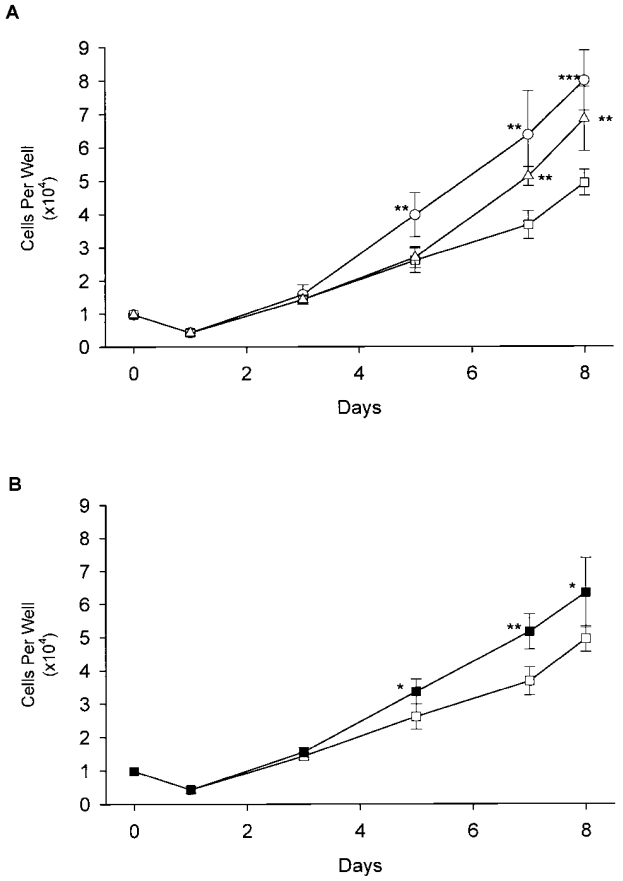

Figure 6. Restoration of Hs 294T cell growth inhibition by removal of antileukinate or the addition of excess MGSA/GROα. Melanoma cell line Hs 294T (2×10^4 cells/well) was cultured in a 24-well tissue culture plate for 1–8 d, and the cell number was counted using a hemocytometer. (*A*) The effect of removal of antileukinate from the cell culture. The cell culture was started in the presence of 50 μM antileukinate. 24 h later, the culture media was replaced using media without antileukinate and the culture was continued in the absence of the peptide (*open triangles*). The cell numbers were compared with those measured when the cells were cultured in the presence (*open squares*) or the absence (*open circles*) of the peptide throughout the culture periods. (*B*) The effect of addition of excess MGSA/GROα to the cell culture. The cell culture was started in the presence of 50 μM antileukinate. 24 h later, the culture media was replaced using media containing antileukinate and 50 nM MGSA/GROα (*closed squares*). The cell numbers were compared with those measured when the cells were cultured in the presence of antileukinate alone (*open squares*). Analysis of variance was used for multiple comparisons of the cell numbers obtained at each culture period. When there was significant difference, the Scheffe's test was performed to establish the significance between two groups. The data significantly different from those with antileukinate (+) were marked: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

ceptors are present in human burn wounds and may act as a mediator for wound repair (21). MGSA/GROα and IL-8 was induced by ultraviolet B radiation in human keratinocyte cell lines (22).

In this study, we examined the binding of α-chemokines to Hs 294T melanoma cell line and found that MGSA/GROα but not IL-8 could bind to the cells. This finding is compatible with the previous study in which the presence of a novel receptor unique for MGSA/GROα on Hs 294T cells was indicated (14). However, recent studies showed that IL-8 receptor B is present in melanoma cells and plays a role in the cell growth (9, 11). IL-8 receptor B messenger RNA and protein expres-

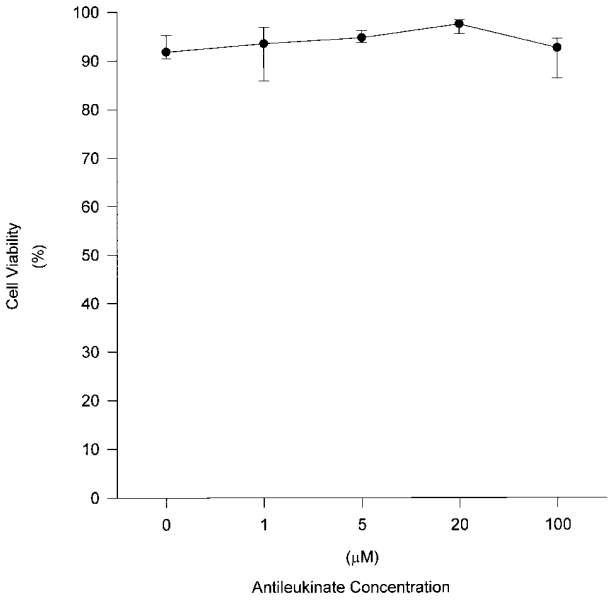

Figure 7. The viability of Hs 294T cells after the culture for 72 h in the presence of antileukinate. Melanoma cell line Hs 294T was cultured in the presence of various concentrations of antileukinate for 72 h. The viability of the cells was measured by means of trypan blue dye exclusion. Median±25% percentile range of the data are shown.

sion was identified in various melanoma cell lines including Hs 294T. The antibody against IL-8 receptor B partially blocked specific binding of MGSA/GROα. Furthermore, addition of $F(ab')_2$ fragments of the anti–IL-8 receptor B or anti-MGSA/GROα monoclonal antibody inhibited serum-independent melanoma cell growth in vitro (11). These studies suggested that, at least a part of, binding of MGSA/GROα to the melanoma cells is mediated by IL-8 receptor B on the surface. As shown in Table I, melanoma cell lines produced IL-8 in $\sim$ 10 times higher concentration than MGSA/GROα. Therefore, our inability to see IL-8 binding to melanoma cells may be explained by there being two types of α-chemokine receptors on melanoma cells, IL-8 receptor B and a unique receptor for MGSA/GROα, while IL-8 receptor B is completely down-regulated by high concentration of IL-8. However, the presence of the novel receptor still remains to be examined, since the receptor has not been demonstrated reproducibly. The alternative explanation of our results is that, although IL-8 receptor B rather than a unique MGSA/GROα receptor is expressed on melanoma cells, IL-8 in the culture made IL-8 receptor B insensitive to IL-8, but not to MGSA/GROα through a certain conformational change of the receptor. The latter explanation is possible since we found that the interaction of IL-8 receptor B with its ligands is not homogeneous (23).

We discovered that a novel hexapeptide, antileukinate, is a potent inhibitor of binding of α-chemokines to the receptors on the neutrophils (12). Antileukinate inhibited the binding of radiolabeled IL-8 with an IC_{50} of 13.7 μM. The activity of the peptide was specific for α-chemokines. Antileukinate also suppressed the binding of radiolabeled macrophage inflammatory peptide-2β (MIP-2β) to human neutrophils, while it did not affect the binding of radiolabeled MIP-1α, leukotriene B_4 or C5a (12). Antileukinate inhibited the enzyme release from neutrophils stimulated by IL-8 with an EC_{50} of 0.8 μM. The mechanism of antileukinate action is not fully defined. In preliminary experiments, however, the peptide bound to human neutrophils, while it did not interact with IL-8 (data not shown). Together with the finding that some part of IL-8 binding to human neutrophil was competitively inhibited by antileukinate (12), these findings suggested that antileukinate interacts with α-chemokine receptors rather than affects the ligand.

In this study, we tested the effect of antileukinate on autostimulatory growth of melanoma cell lines Hs 294T and RPMI-7951. Antileukinate significantly inhibited the binding of MGSA/GROα to these melanoma cell lines. Since Hs 294T and RPMI-7951 secreted MGSA/GROα into the supernatant, we examined the effect of antileukinate on the autostimulatory growth of the cells. When the peptide was added to the culture of melanoma cells, it suppressed the cell growth almost completely at 100 μM. The peptide was more effective on Hs 294T cells than RPMI-7951 cells. The peptide did not affect the growth of the Hep G2 liver cancer cell line, which did not produce MGSA/GROα. The melanoma cell growth inhibition by antileukinate seemed to be associated with specific ability of the peptide to inhibit α-chemokine–receptor interaction rather than nonspecific cell growth inhibition by itself or its metabolites. Antileukinate did not affect the growth of SP2/o mouse myeloma cell line stimulated by murine interleukin-6 (data not shown). Antileukinate did not inhibit the growth of Hep G2 cells which metabolized the peptide in a similar way to Hs 294T cells. The cell viability of Hs 294T in the presence of 100 μM peptide was $>$ 92% as assessed by trypan blue dye exclusion. The peptide did not cause cell lysis of either melanoma cell lines or Hep G2 cells. Furthermore, the removal of the peptide from Hs 294T cell culture or the addition of excess MGSA/GROα to the culture restored the cell growth. These findings suggest that MGSA/GROα is an essential autostimulatory growth factor for melanoma cells and antileukinate inhibits their growth by preventing MGSA/GRO α from binding to its receptors. These studies raise the possibility that melanoma might be treatable with specific inhibitors of growth factors which control melanoma cell growth.

Acknowledgments

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