Angiostatin-mediated Suppression of Cancer Metastases by Primary Neoplasms Engineered to Produce Granulocyte/Macrophage Colony–stimulating Factor

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Summary

We determined whether tumor cells consistently generating granulocyte/macrophage colony–stimulating factor (GM-CSF) can recruit and activate macrophages to generate angiostatin and, hence, inhibit the growth of distant metastasis. Two murine melanoma lines, B16-F10 (syngeneic to C57BL/6 mice) and K-1735 (syngeneic to C3H/HeN mice), were engineered to produce GM-CSF. High GM-CSF (>1 ng/10^6 cells)– and low GM-CSF (<10 pg/10^6 cells)–producing clones were identified. Parental, low, and high GM-CSF–producing cells were injected subcutaneously into syngeneic and into nude mice. Parental and low-producing cells produced rapidly growing tumors, whereas the high-producing cells produced slow-growing tumors. Macrophage density inversely correlated with tumorigenicity and directly correlated with steady state levels of macrophage metalloelastase (MME) mRNA. B16 and K-1735 subcutaneous (s.c.) tumors producing high levels of GM-CSF significantly suppressed lung metastasis of 3LL, UV-2237 fibrosarcoma, K-1735 M2, and B16-F10 cells, but parental or low-producing tumors did not. The level of angiostatin in the serum directly correlated with the production of GM-CSF by the s.c. tumors. Macrophages incubated with medium conditioned by GM-CSF–producing B16 or K-1735 cells had higher MME activity and generated fourfold more angiostatin than control counterparts. These data provide direct evidence that GM-CSF released from a primary tumor can upregulate angiostatin production and suppress growth of metastases.

Key words: angiogenesis • angiostatin • granulocyte/macrophage colony–stimulating factor • metastasis • tumor

The progressive growth and spread of neoplasms are dependent on the formation of adequate vasculature, i.e., angiogenesis (1). The extent of angiogenesis depends on the balance between positive and negative regulating molecules released by both tumor cells and host cells, e.g., lymphoid cells (2–6). Angiogenesis begins with local degradation of the basement membrane of capillaries, followed by invasion of the stroma by underlying endothelial cells in the direction of an angiogenic stimulus. Subsequent to migration, endothelial cells proliferate at the leading edge of a migrating column and then organize into three-dimensional structures to form new capillary tubes (2–6). Interruption of any one of these steps can inhibit angiogenesis (7) and thus metastasis.

Angiostatin is a specific inhibitor of endothelial cell proliferation originally purified from the serum and urine of mice bearing a murine Lewis lung carcinoma (3LL; reference 8). It is an internal fragment of plasminogen containing the first four triple loop disulfide-linked structures, with an apparent molecule mass of 38 kD (8). Angiostatin isolated from serum and urine of mice or from the proteolytic degradation of human plasminogen is a potent inhibitor of angiogenesis and, hence, of tumor growth in vivo (8, 9). Proteolytic degradation of plasminogen by elastase (10), urokinase plasminogen activator in PC3 human prostate carcinoma cells (11, 12), human matrilysin, and human gelatinase B (13) results in the production of angiostatin.

We have reported recently that the production of angiostatin by 3LL tumors growing subcutaneously (s.c. tumors) is associated with the upregulation of murine metalloelastase (MME)[1] in tumor-infiltrating macrophages (10). Further, we concluded that MME induced by GM-CSF can cleave plasminogen to angiostatin (10). Since MME

[1]*Abbreviations used in this paper:* BCE, bovine capillary endothelial cell(s); bFGF, basic fibroblast growth factor; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H, high (levels of GM-CSF–producing); LBS-1, plasminogen lysine–binding site 1; L, low (levels of GM-CSF–producing); MME, macrophage metalloelastase; PEM, peritoneal exudate macrophage(s).

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expression in macrophages is upregulated by GM-CSF (14), we wished to determine whether we could engineer tumor cells to produce high levels of GM-CSF to recruit macrophages and upregulate expression of MME, which would lead to production of angiostatin and thus to inhibition of the outgrowth of cancer metastases.

Materials and Methods

Mice. Athymic Ncr-nu/nu male mice were purchased from the Animal Production Area, National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). Specific pathogen–free female C57BL/6 and C3H/HeN mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained under specific pathogen–free conditions in a facility approved by the American Association for Accreditation of Laboratory Animal Care. The care and experimental procedures were in accordance with institutional guidelines and current regulations and standards of the United States Department of Agriculture, the Department of Health and Human Services, and the National Institutes of Health. The mice were used when they were 8–12 wk old.

Tumor Lines. Metastatic murine Lewis lung carcinoma syngeneic to C57BL/6 mice (3LL-met; obtained from Dr. M. O'Reilly, Harvard Medical School, Boston, MA [8]), B16-F10 GM-CSF, a variant of the metastatic B16-F10 melanoma syngeneic to C57BL/6 mice (15) transduced using an MFG retroviral vector encoding murine GM-CSF (obtained from Dr. D. Pardoll, Johns Hopkins University, Baltimore, MD), metastatic K-1735 M2 melanoma syngeneic to C3H/HeN mice (16), UV-2237M fibrosarcoma syngeneic to C3H/HeN mice (17), and murine RENCA renal carcinoma syngeneic to BALB/c mice (18) were maintained as monolayer cultures in Eagle's MEM supplemented with sodium pyruvate, nonessential amino acids, l-glutamine, vitamin solution (GIBCO BRL, Gaithersburg, MD), and 5% (vol/vol) heat-inactivated fetal bovine serum (FBS) at 10% CO_2/95% air at 37°C. The K-1735 M2 melanoma cells were transfected with the plasmid pcDNA3-GM-CSF encoding for murine GM-CSF driven by human cytomegalovirus promoter (a gift of Dr. W. Yang, Academia Sinica, Taiwan) to derive K-1735 M2 GM-CSF cells. K-1735 M2 cells transfected with pcDNA3 encoding for neomycin resistance gene (K-1735 M2-Neo) served as a control. Cells resistant to 3 wk of continuous exposure to 800 μg/ml G418 were selected.

B16-F10 GM-CSF and K-1735 M2 GM-CSF cells were subcloned by limited dilution. Clones producing low (L) or high (H) levels of GM-CSF were isolated as described below. Bovine capillary endothelial cells (BCE; obtained from Dr. M. O'Reilly [8]) were cultured on gelatinized plastic in DMEM/10% FBS supplemented with 3 ng/ml human basic fibroblast growth factor (bFGF; Genzyme Corp., Cambridge, MA). All cultures were free of mycoplasma and the pathogenic murine viruses reovirus type 3, pneumonia virus of mice, mouse adenovirus, murine hepatitis virus, lymphocyte choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by Program Resources, Inc., NCI-Frederick Cancer Research and Development Center, Frederick, MD).

Measurement of GM-CSF Protein. Tumor cells (10^5) were plated into 30-mm^2 plates in medium containing 5% FBS. After 12 h, the cultures were washed with serum-free medium and then cultured with 2 ml of medium containing 5% FBS for 24 h. The level of GM-CSF in the culture supernatants was determined using an ELISA kit according to the manufacturer's instructions (Quantikine; R&D Systems, Inc., Minneapolis, MN).

In Vivo Studies. Cultures of wild-type and transfected tumor cells in their exponential growth phase were harvested by a brief trypsinization, washed in medium containing 10% FBS, and resuspended in HBSS. Different numbers of cells ($0.5–4 \times 10^5$ in 0.1 ml HBSS) were injected into the subcutis of the right flank of syngeneic or nude mice. When tumors reached 8–10 mm in diameter, unanesthetized syngeneic or nude mice were injected intravenously with 10^5 K-1735 M2 melanoma cells or 10^5 UV-2273M cells. In another set of experiments, nude mice bearing s.c. B16-F10 tumors were anesthetized with methoxyflurane. The tumors in one group of mice were surgically excised, and the area was closed with metal wound clips. The other group underwent a sham surgical procedure. 1 d later, these mice were injected intravenously with 10^5 K-1735 M2 cells. The mice with no s.c. tumors received intravenous injections of 3LL-met; K-1735 M2 cells served as additional controls. The mice were monitored daily, killed 3 wk later, and necropsied. The lungs were weighed and fixed in Bouin's solution. The experimental lung metastases were counted using a dissecting microscope.

To determine whether s.c. tumors that produce GM-CSF can influence the take and growth of tumor cells implanted at another subcutaneous site, we injected B16-F10 GM-CSF cells from 8–10-mm tumors into the subcutis on the right flank of nude mice. K-1735 M2 cells (10^5) were injected into the subcutis of the contralateral flank. Tumor size was determined twice weekly using a caliper. Tumor volume was calculated by the formula, $V = (A \times B^2)/2$, where V = volume, A = long diameter, and B = short diameter.

Collection and Cultivation of Mouse Peritoneal Exudate Macrophages. Peritoneal exudate macrophages (PEM) were collected by peritoneal lavage with HBSS of mice injected intraperitoneally with 1.5 ml thioglycollate broth 4 d previously. The PEM were plated in serum-free medium for 2 h on a 24-well dish at a density of 2×10^5 cells/well. 2 h later, nonadherent cells were removed. The resultant adherent population was 98% pure according to immunologic, morphologic, and phagocytic criteria (19).

Sample Preparations for Elastase and Angiostatin Assays. To collect tumor cell culture supernatants, tumor cells were plated at a density of $2 \times 10^6/75$ cm^2, flasked, and cultured for 48 h in medium containing 5% FBS. The culture supernatant fluids were collected, centrifuged at 3,000 rpm for 10 min to remove intact cells, aliquoted, and stored at −80°C until use. For the elastase and angiostatin assays, PEM were incubated for 24 h with tumor cell culture supernatants diluted in medium containing 5% FBS. The PEM were washed and incubated for another 72 h in 0.5 ml/well of serum-free DMEM/F12 medium in the absence or presence of 100–200 μg/ml human plasminogen purified from human plasma using a lysine–Sepharose 4B column (Pharmacia Biotech AB, Uppsala, Sweden). The culture supernatants were centrifuged at 3,000 *g* for 30 min at 4°C. Elastase or angiostatin activities were assessed as described below.

Elastase Assay. Elastinolytic activity in macrophage-conditioned medium was determined by a method described previously using [^{3}H]$NaBH_4$-labeled elastin as a substrate (20). In brief, the samples were incubated for 16 h at 37°C with 600 μg of the radiolabeled elastin in a reaction buffer (100 mM Tris-HCl, 5 mM $CaCl_2$, 0.2 mg/ml SDS, and 0.006% NaH_3). Released (free-form) ^{3}H-peptide was harvested by centrifugation, and radioactivity was monitored by scintillation counting. Enzyme activity was expressed as cpm per reaction.

Angiostatin Assay. Angiostatin activity was assessed by inhibition of BCE proliferation as described previously (8). In brief, BCE seeded at a density of 1.25×10^4 cells/gelatinized well/0.5 ml DMEM containing 10% FBS were allowed to adhere overnight,

then were rinsed and incubated for 20 min with 0.25 ml/well DMEM/5% FBS or test samples. Additional medium containing bFGF was added to a final concentration of 1 ng/ml. 72 h later, the BCE were harvested by trypsinization and counted using a hemacytometer. Angiostatin activity was determined from the formula for inhibition of BCE proliferation and was expressed as a percentage.

Northern Blot Analysis. Poly(A)$^+$ mRNA was extracted from 10^7 tumor cells at 70% confluence or from tumors growing in vivo using the FastTrack mRNA isolation kit (Invitrogen Corp., San Diego, CA). mRNA was electrophoresed on a 1% denaturing formaldehyde/agarose gel, electrotransferred at 0.6 A to GeneScreen nylon membrane (DuPont-NEN, Boston, MA), and UV cross-linked with 120,000 μJ/cm^2 using a UV Stratalinker 1800 (Stratagene Inc., La Jolla, CA). Hybridization was performed as described previously (21). The nylon filters were washed three times at 55–60°C with 30 mM NaCl/3 mM sodium citrate (pH 7.2)/0.1% SDS (wt/vol). The cDNA probes used in this analysis were a 1.3-kb PstI cDNA fragment corresponding to rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH; reference 22), a 1.7-kb bamboo cDNA fragment of MME (provided by Dr. S.D. Shapiro, Jewish Hospital at Washington University School of Medicine, St. Louis, MO [23]), and a 1.0-kb PstI cDNA fragment of murine GM-CSF. The cDNA fragments were radiolabeled using random primer with α-^{32}P–labeled deoxyribonucleotide triphosphates (Amersham Corp., Arlington Heights, IL). mRNA expression was quantified on a laser densitometer (Ultrascan XL; LKB, Uppsala, Sweden). Each sample measurement was calculated as the ratio of the average area of the GM-CSF or MME to that of the GAPDH transcript and then standardized using the smallest ratio in each group of samples.

Western Blot Analysis. Samples isolated from culture supernatants were mixed with sample buffer (62.5 mM Tris-HCl [pH 6.8], 2.3% SDS, 100 mM dithiothreitol, and 0.05% bromphenol blue), boiled, and separated on 10% SDS-PAGE. The protein was transferred onto 0.45-μm nitrocellulose membranes. The filter was blocked with 3% BSA in Tris-buffered saline (20 mM Tris-HCl [pH 7.5], 150 mM NaCl), probed with antibody against plasminogen lysine-binding site (LBS)-1 (1 μg/ml) in Tris-buffered saline containing 0.1% Tween 20, incubated with a second antibody in the buffer, and visualized by the enhanced chemiluminescence Western blotting detection system (24).

Immunohistochemistry. The presence of macrophages in tumors was identified by immunohistochemistry using the macrophage-specific scavenger receptor antibody (25). At necropsy, tumors were cut into 5-mm^3 pieces, placed in OCT compound (Miles Inc., Elkhart, IN), and snap frozen in liquid nitrogen. Frozen sections (8–10 μm) were air-dried, fixed with cold acetone, rinsed with PBS, and treated with 3% hydrogen peroxide in methanol (vol/vol). The treated slides were incubated in a blocking solution (5% normal human serum/1% normal goat serum in PBS [vol/vol]) and then for 18 h with a rat polyclonal antibody against scavenger receptor (Serotec Ltd., Kidlington, Oxford, UK) at a 1:70 dilution at 4°C in a humidified chamber. The sections were then rinsed and incubated, first with the blocking solution and then with a peroxidase-conjugated anti–rat antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a 1:100 dilution. Positive reactions were visualized by incubating the slides for 1–5 min with Stable DAB (Research Genetics, Huntsville, AL), followed by counterstaining with Mayer's hematoxylin (Research Genetics). The slides were dried and mounted with Universal mount (Research Genetics). Images were digitized using a color video camera (3CCD; Sony Corp., Tokyo, Japan) and a personal computer equipped with image analysis software (Optimas Corp., Bothell, WA).

Purification of Angiostatin from Murine Serum. Mice were bled from the lateral tail vein. Serum diluted 1:2 to 1:10 with PBS was incubated overnight with lysine–Sepharose 4B (Pharmacia Biotech AB) at 4°C. After washing with PBS and 0.3 M phosphate buffer containing 3 mM EDTA (pH 7.4), lysine-binding proteins were eluted with 0.2 M aminocaproic acid (pH 7.4). The aminocaproic acid in the eluates was removed using a spin column (Microcon 10; Amicon, Inc., Beverly, MA), and the proteins were resuspended in 20 mM Tris-HCl (pH 7.6).

Statistical Analysis. The significance of the in vitro data, lung weight, and tumor volume was analyzed by the Student's *t* test (two-tailed). The difference in the number of lung metastases between groups was compared using the Mann-Whitney U test.

Results

Establishment of GM-CSF–producing Murine Melanoma Cell Lines. In the first set of experiments, we cloned GM-CSF–transduced B16-F10 cells (20). Several clones producing 60–75 ng GM-CSF/10^6 cells/24 h were combined, and the line was designated B16-H (high). Three clones producing <10 pg GM-CSF/10^6 cells/24 h were combined to yield the B16-L (low) line. GM-CSF–transfected K-1735 M2 cells were also cloned (see Materials and Methods). Several clones producing 1–2 ng GM-CSF/10^6 cells/24 h were combined to yield the K-1735-H line. Several clones producing <10 pg GM-CSF/10^6 cells/24 h were combined to yield the K-1735-L line. The level of GM-CSF mRNA directly correlated with the level of protein (Fig. 1, *top* and *bottom*). Southern blot analysis indicated that recombinant GM-CSF DNA was intact in all the cell lines (data not shown).

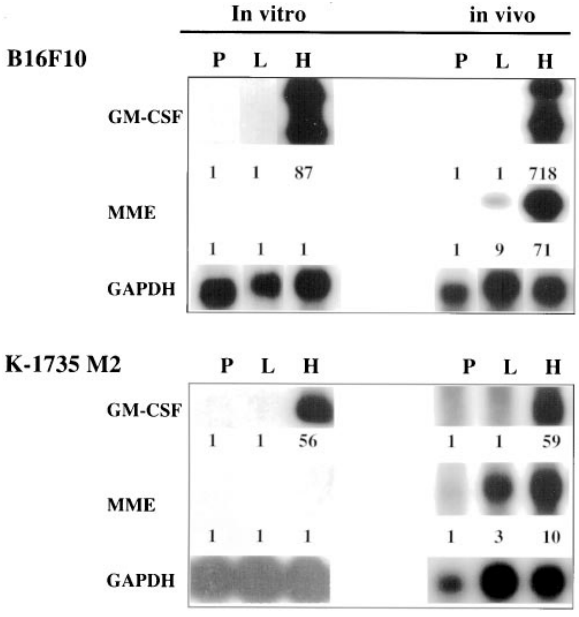

Figure 1. Northern blot analysis. Polyadenylated mRNA (2 μg/lane) from cultured cells or tumors of B16-F10 (*top*) or K-1735 M2 (*bottom*) was separated, blotted, and hybridized with cDNA fragments corresponding to murine GM-CSF, MME, or rat GAPDH. *P*, Parental B16-F10 or K-1735 M2; *L*, B16-L or K-1735-L; *H*, B16-H or K-1735-H.

The production of GM-CSF did not alter the in vitro growth of the tumor cells. In contrast, tumorigenicity and rate of growth of s.c. tumors (in syngeneic or nude mice) inversely correlated with production of GM-CSF. B16-F10 parental or B16-L cells produced 1,800-mm^3 tumors by day 25, whereas the B16-H tumors measured 321 mm^3. Similar results were obtained with the K-1735 melanoma. 3 wk after subcutaneous inoculation, K-1735 M2 parental, K-1735-L, and K-1735-H cells produced 900-, 900-, and 310-mm^3 tumors, respectively.

Macrophage Infiltration into s.c. Tumors and Expression of MME. Immunohistochemical staining of cryostat sections using an mAb against the murine macrophage-specific scavenger receptor revealed that the density of tumor-infiltrat-

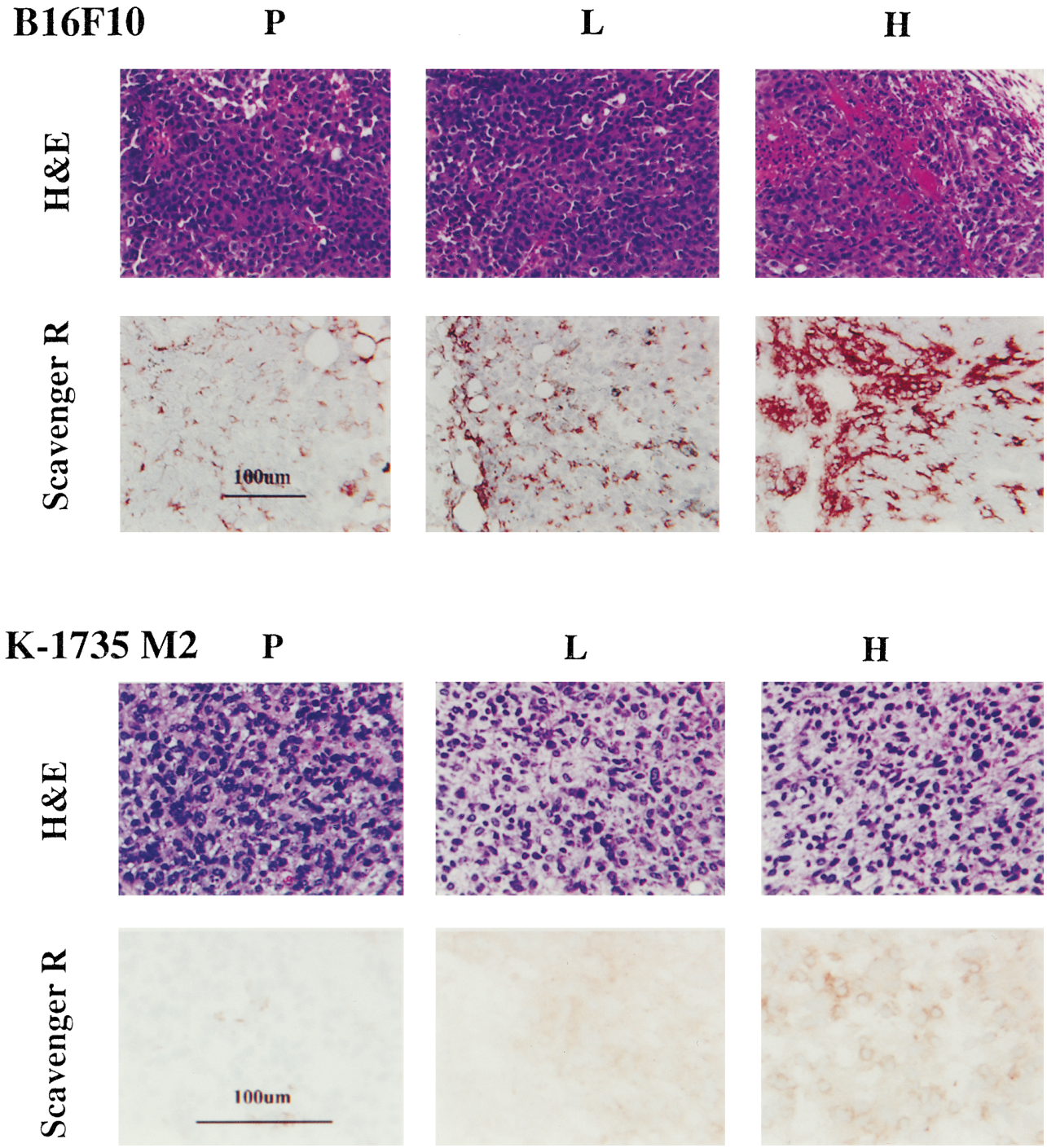

Figure 2. Macrophage infiltration into s.c. tumors. B16-F10 (*top*) or K-1735 M2 (*bottom*) tumors (8–10 mm in diameter) were resected, fixed in formalin for hematoxylin and eosin staining (*H&E*) or in liquid nitrogen for immunohistochemical staining using antimacrophage-specific scavenger receptor antibody (reference 42).

Table 1. *Suppression of Experimental Lung Metastasis by GM-CSF–producing s.c. Tumors*

Tumors	Challenge	Lung metastases		Lung weight
		Median	Range	(*mg, mean ± SD*)
Experiment 1 (C57BL/6 mice)				
None, control	3LL-met	56	29–84	978 ± 146
B16-L	3LL-met	43	31–60	880 ± 92
B16-H	3LL-met	17*	3–31	310‡ ± 57
Experiment 2 (C3H/HeN mice)				
None, control	UV-2237M	39	25–56	348 ± 52
K-1735-L	UV-2237M	27	14–36	325 ± 39
K-1735-H	UV-2237M	5*	0–13	237[b] ± 36

B16-L (5×10^4), B16-H (4×10^5), K-1735-L (10^5), or K-1735-H (10^5) cells were injected subcutaneously into C57BL/6 mice ($n = 10$) or C3H/HeN mice ($n = 5$). When the s.c. tumors reached 8–10 mm in diameter, 10^5 3LL-met tumor cells or 2×10^5 UV-2237M cells were injected intravenously. Mice were killed 26 d later and necropsied. The lungs were weighed and fixed in Bouin's solution, and the experimental lung metastases (tumor colonies) were counted under a dissecting microscope.
*$P < 0.01$, compared with control (Mann-Whitney test).
‡$P < 0.05$, compared with control (Student's *t* test, two-tailed).

ing macrophages in B16-H (Fig. 2, *top right*) and K-1735-H (Fig. 2, *bottom right*) tumors was threefold that in parental (GM-CSF–negative) tumors. Specifically, the average number of macrophages/100× field in the B16-F10, B16-L, and B16-H tumors was 23, 21, and 85, respectively. In the K-1735 M2, K-1735-L, and K-1735-H s.c. tumors, the average number of macrophages/100× field was 11, 31, and 65, respectively. Northern blot analysis indicated that

Table 2. *Nonimmunologic Suppression of Experimental Lung Metastasis by GM-CSF–producing B16 Melanoma Tumors Growing Subcutaneously in Nude Mice*

Tumors	Challenge	Tumor	Lung metastases		Lung weight
			Median	Range	(*mg, mean ± SD*)
Experiment 1					
None, control	—	K-1735 M2	164	148–289	1040 ± 134
B16-L	Sham surgery	K-1735 M2	163	139–186	898 ± 201
B16-L	Resection	K-1735 M2	171	146–201	1020 ± 268
B16-H	Sham surgery	K-1735 M2	46*	26–61	322‡ ± 81
B16-H	Resection	K-1735 M2	185	173–214	967 ± 268
Experiment 2					
None, control	—	3LL-met	53	21–84	995 ± 198
B16-L	Sham surgery	3LL-met	34	29–51	813 ± 104
B16-L	Resection	3LL-met	48	36–62	971 ± 189
B16-H	Sham surgery	3LL-met	16*	2–19	254‡ ± 43
B16-H	Resection	3LL-met	50	33–63	877 ± 140

Nude mice were injected subcutaneously with 5×10^4 B16-L or 4×10^5 B16-H cells. When the tumors reached 8–10 mm in diameter, the mice were anesthetized and the tumors were resected from one group of mice ($n = 20$), and the other group underwent sham surgery. 1 d later, the mice were injected intravenously with 10^5 K-1735 M2 or 10^5 3LL-met cells. The mice were killed 3 wk later and necropsied. The lungs were weighed and fixed in Bouin's solution, and the experimental lung metastases (tumor colonies) were counted under a dissecting microscope.
*$P < 0.01$, compared with control (Mann-Whitney test).
‡$P < 0.01$, compared with control (Student's *t* test, two-tailed).

tumors produced by B16-H and K-1735-H cells, but not B16-L, K-1735-L, or their parental cells, expressed high levels of GM-CSF and MME mRNA (Fig. 1, *top* and *bottom*, in vivo). These results suggest that GM-CSF released by tumor cells recruits macrophages and induces MME gene expression in the tumor-infiltrating macrophages.

Suppression of Distant Tumor Growth by GM-CSF–producing s.c. Tumors. We next determined whether the cells engineered to produce GM-CSF could affect the growth of distant tumors. Because we used tumors of different origins for the local (s.c.) and secondary (metastases) challenges, we could rule out the contribution of T cell–mediated specific immune response in suppression of secondary tumors (26–28). Specifically, B16-L and B16-H (syngeneic to C57BL/6 mice) or K-1735-L and K-1735-H cells (syngeneic to C3H mice) were inoculated subcutaneously into syngeneic recipients. When the tumors reached 8–10 mm in diameter, 3LL-met (syngeneic to C57BL/6 mice) or UV-2237M (syngeneic to C3H mice) cells were injected intravenously into the mice bearing B16 or K-1735 tumors, respectively. In both tumor systems, the s.c. tumors produced by cells releasing high amounts of GM-CSF (but not parental or low GM-CSF–producing cells) significantly suppressed ($P < 0.01$) the growth of experimental lung metastasis by 3LL or UV-2237M cells (Table 1).

Next, we injected B16-L or B16-H cells into the subcutis of nude mice. When the tumors reached 10 mm in diameter, the mice were anesthetized. One group ($n = 10$) underwent resection of the s.c. tumors, and the other ($n = 10$) underwent sham surgery. 1 d later, the mice were injected intravenously with 3LL-met K-1735 M2 cells. The mice were killed 21 d later and necropsied, and the lung metastases were counted under a microscope. The data shown in Table 2 demonstrate that an intact s.c. B16-H (but not B16-L) tumor significantly reduced the median number of lung metastases and their size (lung weight) produced by both 3LL-met and K-1735 M2 cells. In contrast, the median number or size of lung metastases in mice whose primary/s.c. tumor was resected did not differ from control mice.

The B16-H tumors in the subcutis also suppressed the growth of K-1735 M2 cells implanted into the contralateral flank of nude mice (Fig. 3). By day 20 after inoculation of the second tumor, the volume of the K-1735 M2 tumors was 936 (132, 724 ± 143, and 288 ± 12 mm^2 in mice bearing a primary B16-P, B16-L, and B16-H s.c. tumor, respectively; $P < 0.01$).

Angiostatin in the Serum of Tumor-bearing Mice. A previous report from our laboratory concluded that MME induced by GM-CSF can cleave plasminogen to angiostatin (10). To correlate the presence of angiostatin with suppression of metastases, we collected serum from normal mice and mice with B16-L and B16-H s.c. tumors. The sera were enriched for lysine-binding protein by passing through a lysine–Sepharose 4B column, and the eluents were separated on 10% SDS-PAGE, blotted, and probed with an mAb against human LBS-1. Western blotting analysis demonstrated that serum from both normal mice and mice bearing B16-L tumors contained 38-kD protein(s) that reacted with the antibody (Fig. 4). The same band with significantly higher intensity was found in the serum of mice bearing B16-H tumors. These results suggest that angiostatin is present in normal mice and that its production is upregulated in mice bearing B16-H but not B16-L tumors.

Generation of Angiostatin by Cultured Macrophages. PEM were treated for 24 h with media conditioned by wild-type or GM-CSF gene–engineered B16 or K-1735 cells. The cells were then washed and incubated for an additional 72 h in serum-free medium in the absence or presence of human plasminogen. The resultant supernatants were assessed for elastase (Fig. 5 *A*) and angiostatin (Fig. 5 *B*) activity. The level of elastinolytic activity constitutively secreted by mouse PEM was not altered by treatment with media conditioned by B16-P, K-1735-P, B16-L, or K-1735-L cells. In contrast, PEM elastinolytic activity reached 1.5–2-fold the control levels with PEM incubated with media conditioned by B16-H or K-1735-H (Fig. 5 *A*). Significantly higher angiostatin activity (determined by a bioassay using growth inhibition of BCE) was found in supernatants of PEM with higher MME activity and plasminogen. Specifically, the mixture derived from control PEM or PEM conditioned by medium of control tumor cells inhibited growth of BCE by 10–15%, whereas the mixture from PEM incu-

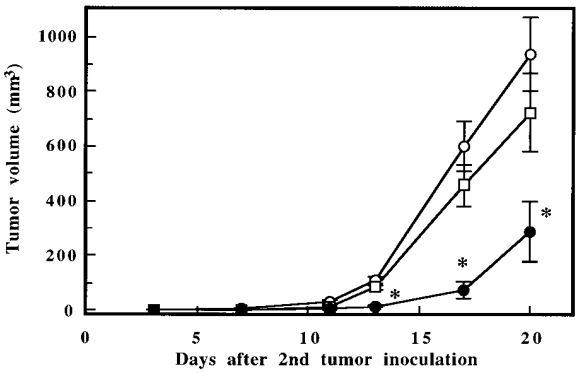

Figure 3. Suppression of the growth of K-1735 M2 cells implanted subcutaneously by distant s.c. B16-H tumors. Nude mice ($n = 10$) were injected subcutaneously with 4×10^5 B16-L (*open squares*) or 5×10^4 B16-H (*filled circles*) cells. When the tumors reached 8–10 mm in diameter, K-1735 M2 cells (10^5/mouse) were injected subcutaneously into the contralateral flank of tumor-bearing mice or normal mice (*open circles*) serving as additional controls. Tumor size was measured twice weekly using a caliper, and tumor volume was calculated by the formula $V = (A \times B^2)/2$.

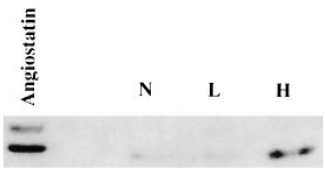

Figure 4. Identification of angiostatin in the serum. Angiostatin in the serum of control or tumor-bearing mice was purified by a lysine–Sepharose 4B column, separated on 10% SDS-PAGE, blotted, and identified using an mAb against plasminogen LBS-1. *N*, Normal mice; *L*, mice bearing B16-L tumors; *H*, mice bearing B16-H tumors.

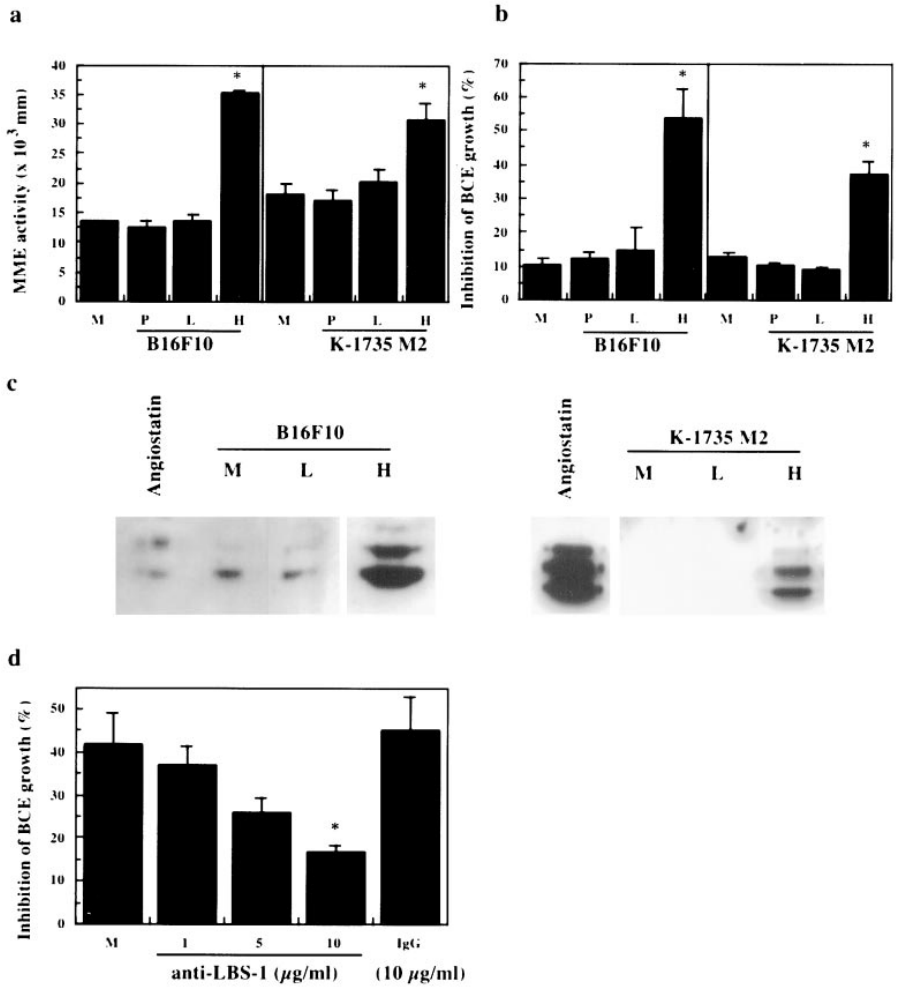

Figure 5. In vitro generation of MME and angiostatin. Mouse PEM were treated for 24 h in medium conditioned by B16-F10 or K-1735 M2 cells. The cultures were briefly rinsed and incubated for 72 h in serum-free DMEM/F12 medium in the absence (*a*) or presence (*b*) of 100 μg/ml of human plasminogen. MME activity (*a*) and angiostatin activity (*b*) in the culture supernatants were determined as described above. Plasminogen fragments in the supernatants were identified by Western blot analysis using an antibody against plasminogen LBS-1 (*c*). The angiostatin assay was conducted in the presence of anti–LBS-1 (1, 5, or 10 μg/ml) or control IgG (10 μg/ml) (*d*). *M*, Medium; *P*, parental B16-F10 or K-1735 M2; *L*, B16-L or K-1735-L; *H*, B16-H or K-1735-H.

bated with medium conditioned by cells releasing high amounts of GM-CSF inhibited BCE growth by 40–55% (Fig. 5 *B*). The growth inhibition of BCE was neutralized by treatment of the supernatants with antibodies against LBS-1 but not by an isotype-matched control immunoglobulin (Fig. 5 *C*). Western blot analysis using the antibody to LBS-1 confirmed that the mixtures derived from PEM treated by medium conditioned by B16-H or K-1735-H cells contained significantly higher levels of the 38-kD protein (Fig. 5 *D*).

Discussion

The growth of metastases can accelerate subsequent to resection of some primary neoplasms (29, 30). This accelerated growth can be found in both autochthonous human neoplasms and experimental rodent tumors, and is independent of specific immune response (31–35). Sustained tumor growth depends on an adequate supply of nutrients and thus on angiogenesis (2, 36–38), which has led to efforts to block the angiogenic pathway. Recent studies have concluded that some primary neoplasms can induce the production of a breakdown product from plasminogen called angiostatin (8) that specifically inhibits proliferation of vascular endothelial cells (8, 10). By thus inhibiting the development of adequate vasculature, circulating angiostatin can inhibit the growth of metastases and contribute to dormancy of metastatic tumor cells (9, 12, 39–41). One mechanism for the in vivo production of angiostatin involves the upregulation of MME in macrophages that infiltrate tumor cells that produce GM-CSF (10). Whether GM-CSF–negative tumors engineered to produce GM-CSF could thus generate production of angiostatin by infiltrating macrophages remained unclear.

The present results demonstrate that s.c. tumors of B16 melanoma and K-1735 melanoma cells engineered to produce high amounts of GM-CSF were infiltrated by macrophages, whereas parental (wild-type) or cells producing low levels of GM-CSF were not. The production of GM-CSF directly correlated with infiltrating macrophages' MME activity and production of circulating angiostatin and, hence, growth inhibition of tumors implanted at a distant subcutaneous site or growing as lung metastases. In vitro analysis indicated that treatment of macrophages with media conditioned by cells producing a high level of GM-CSF increased the secretion of MME and production of angiosta-

tin from plasminogen. These data confirmed our previous findings (10, 14), and provide direct evidence that GM-CSF can augment angiostatin production by upregulating expression of MME in tumor-infiltrating macrophages.

Tumor cells producing a high level of GM-CSF had decreased tumorigenicity in both syngeneic and nude mice compared with control cells or cells producing low levels of GM-CSF. The reduced tumorigenicity was not restricted to that produced by tumors injected in the subcutaneous site. B16 or K-1735 cells producing high levels of GM-CSF also had a reduced incidence of experimental lung metastasis (data not shown). The decrease in tumorigenicity could well be due to tumor-infiltrating macrophages. First, the density of tumor-infiltrating macrophages directly correlated with production of GM-CSF. Second, preliminary analysis indicates that tumor cells producing high levels of GM-CSF were more susceptible to lysis mediated by macrophages than low GM-CSF–producing counterparts (data not shown).

The production of GM-CSF by tumor cells has been correlated with stimulation of antigen presenting cells and induction of tumor-specific T cell–mediated immunity (26–28). However, specific immunity cannot account for the present results. We base this conclusion on results of two major experiments. First, in many of the studies, s.c. B16 tumors (producing GM-CSF) inhibited the outgrowth of syngeneic but non–cross-reactive 3LL carcinoma cells. Second, lung metastasis was inhibited in athymic nude mice when GM-CSF–producing s.c. tumors were coinjected with metastatic allogeneic tumor cells.

In summary, we provide direct evidence that upregulation of MME expression in macrophages by GM-CSF produced by local tumors can lead to generation of angiostatin and, hence, growth suppression of distant metastases. Recent data indicate that angiostatin can also be generated by reduction and proteolysis of plasmin (11, 12) and by cleavage of plasminogen by gelatinase B and matrilysin (13). Regardless of the biochemical mechanism, our data suggest that chronic administration of GM-CSF or transduction of the GM-CSF gene into tumor or normal tissue can be used to treat cancer metastasis.

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