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 metastases. 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/10⁶ cells)- and low GM-CSF (<10 pg/10⁶ 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) in tumor-infiltrating macrophages (10). Further, we concluded that MME induced by GM-CSF can cleave plasminogen to angiostatin (10). Since MME
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 M ME, which would lead to production of angiostatin and thus to inhibition of the outgrowth of cancer metastases.

Materials and Methods

Mice. Athymic N cr-nu/nu male mice were purchased from the Animal Production Area, National Cancer Institute-Frederick Cancer Research Facility (Frederick, M D). 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. D. Pardoll, Johns Hopkins University, Baltimore, MD), metastatic K-1735 M2 melanoma syngeneic to C3H/HeN mice (16), and murine M2 melanoma syngeneic to C3H/HeN 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 (obtained from Dr. M. P. Also, the MMV-2237M, Johns Hopkins University, Baltimore, MD), metastatic K-1735 M2 melanoma syngeneic to C3H/HeN mice (16), and murine RENCA renal carcinoma syngeneic to BALB/c mice (18) were fibrosarcoma syngeneic to C57BL/6 mice (15) transduced using an MFG retroviral vector encoding murine GM-CSF, a variant of the metastatic B16-F10 melanoma syngeneic to C57BL/6 mice (3LL-met) (obtained from Dr. M. Pardoll, Johns Hopkins University, Baltimore, MD).

Regulation of Angiostatin Production by GM-CSF

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 re-suspended in HBSS. Different numbers of cells (0.5–4 × 10^6 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, anaesthetized syngeneic or nude mice were injected intravenously with 10^6 K-1735 M2 melanoma cells or 10^6 UV-2237M cells. In another set of experiments, nude mice bearing s.c. B16-F10 tumors were anesthetized with methoxyfluorane. 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. One day later, these mice were injected intravenously with 10^6 K-1735 M2 cells. The mice with no sc. 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 (10^6) were injected into the subcutis on the contralateral flank. Tumor size was determined twice weekly using a caliper. Tumor volume was calculated by the formula, V = (A × B^2)/2, where A = tumor diameter, B = diameter perpendicular to A, and C = height of pyramid.

Expression and secretion of murine angiostatin in macrophage-conditioned medium was determined by a method described previously using [3H]NaBH4 labeled elastin as a substrate (18). 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 CaCl2, 0.2 mg/ml SDS, and 0.006% NaOH). R released (free-form) [3H]-peptide was harvested by centrifugation, and radioactivity was measured 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 DM EM/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 hemacy-

ometer. Angiostatin activity was determined from the formula for

polarity and was expressed as a percentage.

Northern Blot Analysis. Poly(A)- mRNA was extracted from 10² tumor cells at 70% confluence from or from tumors growing in vivo using the FastTrack mRNA isolation kit (Invitrogen Corp., San Diego, CA). mRNA was electrophoresed on a 1% denaturing formamide/agarose gel, electrotransferred at 0.6 A to Gene-

chip nylon membrane (DuPont-NEN, Boston, MA), and UV cross-linked with 120,000 μJ/cm² 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 1.0-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-32P-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 area of the average area of the GM-CSF or MME to that of the GAPDH transcript and then standardized using the smallest area in each group.

Western Blot Analysis. Samples isolated from culture superna-
tants were mixed with sample buffer (62.5 mM Tris HCl [pH 6.8], 0.1% SDS, 100 mM dithiothreitol, and 0.05% bromophenol 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) for 1 h. The filter was then incubated with antibody against plasminogen lysine-binding site (LBS)-1 (1 μg/ml) in Tris-buffered saline containing 0.1% SDS (wt/vol) for 1 h. The filter was washed three times with PBS and 0.1% Tween 20, incubated with a second antibody conjugated to horseradish peroxidase, and then 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 1 h with a rat polyclonal antibody against scavenger receptor (Serotec Ltd., Kidlington, Oxford, U.K.) 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 hema-

toxyl (Research Genetics). The slides were dried and mounted with Universal mount (Research Genetics). Images were digi-
tized using a color video camera (3CCD; Sony Corp., Tokyo, Japan) and a personal computer equipped with image analysis soft-

ware (Optimas Corp., Bothell, WA).

Purification of Angiostatin from Mouse 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 Bio-
tech 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 amin-
ocaproic 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 be-
tween groups was compared using the Mann-Whitney U test.

Results

Establishment of GM·CSF-Producing M urine 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/10⁶ cells/24 h were combined, and the line was designated B16-H (high). Three clones producing <10 pg GM·CSF/10⁶ 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/10⁶ cells/24 h were combined to yield the K-1735-H line. Several clones producing <10 pg GM·CSF/10⁶ 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 recombiant 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-mm³ tumors by day 25, whereas the B16-H tumors measured 321 mm³. 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-mm³ 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).
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 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) |
| N one, 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) |
| N one, control | U V-2237M | 39 | 25–56 | 348 ± 52 |
| K-1735-L | U V-2237M | 27 | 14–36 | 325 ± 39 |
| K-1735-H | U V-2237M | 5* | 0–13 | 237‡ ± 36 |

B16-L (5 × 10⁴), B16-H (4 × 10⁵), K-1735-L (10⁶), or K-1735-H (10⁶) 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, 10⁵ 3LL-met tumor cells or 2 × 10⁵ 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 < 0.01, compared with control (Student’s t test, two-tailed).

Nude mice were injected subcutaneously with 5 × 10⁴ B16-L or 4 × 10⁵ 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 10⁵ K-1735 M2 or 10⁵ 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).

| 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 |
| N one, 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 |
| N one, 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 × 10⁴ B16-L or 4 × 10⁵ 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 10⁵ K-1735 M2 or 10⁵ 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).
The 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 cell lines. We next determined whether the cells engineered to produce GM-CSF could affect the growth of distant tumors. Because we used tumor cells 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 \( 936 (132, 724 \pm 143, \text{ and } 288 \pm 12 mm^2) \) 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 and finally probed with a mouse anti-LBS-1. Western blotting analysis demonstrated that serum from both normal mice and mice bearing B16-L and B16-H s.c. 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 up-regulated 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 elastolytic 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 elastolytic activity reached 1.5–2-fold the control level 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 \( 4 \times 10^4 \) B16-L (open squares) or \( 5 \times 10^4 \) B16-H (filled circles) cells. When the tumors reached 8–10 mm in diameter, K-1735 M2 cells \( 10^3 \) 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 \( V = (A \times B^3)/2 \).

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.
bated with medium conditioned by cells releasing high amounts of GM-CSF inhibited BCE growth by 40–55\% (Fig. 5B). 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. 5C). 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. 5D).

**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 angiostatin.
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, many of the studies, so 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 conjoined 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.

The authors thank Walter Pagel for his critical editorial review, and Lola López for expert assistance in the preparation of this manuscript. We also thank Dr. Corazon D. Bucana and Mrs. Donna Reynolds for technical assistance with immunohistochemical staining.

This work was supported in part by grant CA16672 from the Cancer Center Support Core, and grant R35-CA-42107 from the National Cancer Institute, National Institutes of Health.

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Received for publication 4 May 1998.

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