Granulocyte Macrophage Colony-Stimulating Factor Inhibits Breast Cancer Growth and Metastasis by Invoking an Anti-Angiogenic Program in Tumor-Educated Macrophages


Abstract

Tumor-educated macrophages facilitate tumor metastasis and angiogenesis. We discovered that granulocyte macrophage colony-stimulating factor (GM-CSF) blocked macrophages vascular endothelial growth factor (VEGF) activity by producing soluble VEGF receptor-1 (sVEGFR-1) and determined the effect on tumor-associated macrophage behavior and tumor growth. We show GM-CSF treatment of murine mammary tumors slowed tumor growth and slowed metastasis. These tumors had more macrophages, fewer blood vessels, and lower oxygen concentrations. This effect was sVEGFR-1 dependent. In situ hybridization and flow cytometry identified macrophages as the primary source of sVEGFR-1. These data suggest that GM-CSF can re-educate macrophages to reduce angiogenesis and metastases in murine breast cancer. [Cancer Res 2009;69(5):2133–40]

Introduction

Cells in the tumor environment facilitate tumor growth and spread. Tumor cells influence endothelial cells, macrophages, T cells, and fibroblasts to evade host defenses, undergo angiogenesis, and produce factors that promote growth, survival, and metastases (1). Tumors grow through signals elicited from cells in their microenvironment. For instance, some tumors down-regulate immune surveillance molecules to avoid attack by T cells and natural killer cells (2, 3). Some secrete growth factors that stimulate blood vessel formation (4). Other tumors stop making molecules that maintain cell-cell interactions (5). Changes tumors impose on surrounding cells are called “tumor education” (6), and often represent an inappropriate triggering of developmental programs within the tumor cells (7).

One type of immune cell, the macrophage, plays an important role in normal breast tissue development. Macrophage activity, stimulated by macrophage colony-stimulating factor (M-CSF), is essential for normal breast development (8). In breast tumors, macrophages constitute up to 35% of the infiltrating inflammatory cells (9). These tumor-associated macrophages (TAM) produce factors that facilitate tumor invasion and angiogenesis, such as matrix metalloproteinases (10) and vascular endothelial growth factor (VEGF; ref. 11).

The cytokine milieu in the tumor microenvironment dictates macrophage behavior. Many breast tumors secrete M-CSF, which is expressed in over 70% of human breast cancers (12). Serum M-CSF levels correlate with tumor size, metastasis, and poor outcomes in humans (13, 14). Mice deficient in M-CSF are protected against breast tumor metastasis, and re-expressing M-CSF solely in the breast tissue restores metastatic activity (15). This effect likely involves both an M-CSF/EGF paracrine loop between tumors and macrophages (16) and M-CSF–induced VEGF production (11), inducing angiogenesis (17).

In sharp contrast, granulocyte M-CSF (GM-CSF)–stimulated monocytes exhibit antitumor behavior. GM-CSF enhances macrophage antigen presentation and immune responsiveness (18). We showed that GM-CSF stimulates monocytes to secrete VEGF receptor-1 (sVEGFR-1), which binds and inactivates VEGF and blocks angiogenesis (19). Angiogenesis within the tumors is necessary for tumor progression, as tumors cannot grow beyond a few cubic millimeters without blood vessel formation to supply oxygen and nutrients (20, 21).

Recent studies illustrate the importance of sVEGFR-1 in blocking cancer progression. For example, low intratumor sVEGFR-1 and high total VEGF are associated with poor disease-free and overall survival (22). Toi and colleagues (23) found that tumors with 10-fold more sVEGFR-1 than VEGF have a favorable prognosis. Other studies show similar findings for patients with colorectal cancer (24), glioblastoma (25), and acute myeloid leukemia (26).

These observations led us to speculate that macrophage behavior was manipulated in vivo by GM-CSF. We wanted to know whether the TAM phenotype was reversed by GM-CSF within the tumor microenvironment. We show that intratumor GM-CSF injections reversed some of the effects of tumor education and induced an antitumor phenotype in TAMs.

Materials and Methods

Mice. PyMT transgenic mice were purchased from The Jackson Laboratory. Mammary tumors from PyMT transgenics were removed and orthotopically injected into normal FVB female mice for these studies.

Tumor injections. MET-1 tumor cells were cultured in DMEM containing 10% fetal bovine serum, 10 μg/ml insulin, and 5 ng/ml rhEGF. These cells were resuspended in DMEM medium at 500,000 cells/100 μL. The cells were orthotopically injected into the number four mammary fat pads of normal female FVB mice (allografts).

Treatment study. After tumors became palpable, mice were randomly assigned to treatment groups. PBS or 100 ng mGM-CSF in 50 μL was administered directly into the tumor. For longer time point studies, mice...
were treated until their tumors reached 2 cm in diameter. For short time point studies, seven treatments were administered (thrice per week). Tumor dimensions and mouse weight were measured weekly for long time point studies or at each treatment for shorter studies. For studies analyzing the effect of neutralizing sVEGFR-1 in combination with GM-CSF treatment, tumors were orthotopically injected. Either PBS, 100 ng GM-CSF, 100 ng GM-CSF + 4 μg anti-VEGF receptor-1 neutralizing antibody (R&D Systems; AF471), 100 ng GM-CSF + 4 μg isotype IgG control (goat), or 4 μg anti-VEGF receptor-1 neutralizing antibody alone in 50 μL was injected directly into the tumors.

Electron paramagnetic resonance oximetry. Lithium octa-n-butoxy 2,3-naphthalocyanine (LiNc-BuO) microcrystals were a gift from Dr. Periannan Kuppusamy, The Ohio State University. Ten-microgram microcrystals were resuspended in 500 μL DMEM. Twenty-five microliters of this suspension were added to 5 × 10⁶ PyMT cells for each 100 μL injection. Oxygen measurements were performed immediately, weekly, and upon sacrifice using in vivo electron paramagnetic resonance (EPR) oximetry. Measurements of tumor oxygenation were performed using an L-band in vivo EPR spectrometer (L-band; Magnetech). Mice were placed in a right, lateral position with their tumor close to the surface coil resonator. EPR spectra were acquired as single 30-s scans. The instrument settings were as follows: incident microwave power, 4 mW; modulation amplitude, 180 mG; modulation frequency, 100 kHz; receiver time constant, 0.2 s. The peak-to-peak width of the EPR spectrum was used to calculate pO₂ using a standard calibration curve. Three-dimensional imaging was performed as described previously (27).

Evaluation of angiogenesis. Texas red–conjugated dextran (molecular weight 70,000; Molecular Probes) was prepared to 6.2 mg/mL in PBS and injected via the tail vein at 20 μg/g of mouse body weight. The mice were sacrificed 5 min after administration. Tumor sections were analyzed blindly for fluorescence using a confocal microscope with a ×10 objective lens and image insets with a ×5 zoom. Functional blood vessels (Texas red–dextran positive) were identified and quantified using Adobe Photoshop CS2.

Evaluation of necrosis. Necrotic tissue within the tumors of mice was evaluated as described (Achilles and colleagues, 2001). Necrosis was quantified in a blinded manner using histogram analysis in Adobe Photoshop CS2 software for the presence of green pixels.

Tumor metastases. The lungs from mice implanted with tumor cells and treated with GM-CSF or PBS were removed, insufflated, fixed in paraformaldehyde, and stained with hematoxylin. Tumor cell metastases were evaluated by subjecting the lungs to Bright Field light under a stereomicroscope. Each tumor incident was counted in a blinded manner.

In situ hybridization. Our protocol for detection of RNAs by in situ hybridization has been previously published (28).

Immunohistochemistry. To identify cells producing VEGF or sVEGFR-1 mRNA, serial sections of the tumors were cut and stained for F4/80+ cells. Our immunohistochemical detection protocol is published (28). For identification of cell infiltrates in response to treatment, mammary tumors were stained to identify F4/80+ (macrophages) and 7/4 (neutrophils) as well as H&E. Total cell influx was analyzed by digital images of the entire tumors and quantified using histogram analysis in Adobe Photoshop CS2 software for brown pixels.

Immunofluorescence. Macrophage phenotype was determined on tumor sections stained. With primary antibodies: goat anti-mouse arginase I (Santa Cruz; sc-18351) and rabbit anti-mouse iNOS (Abcam; ab15323), at 1:200 dilutions. Secondary antibodies used were goat anti-goat–Alexa 488 and chicken anti-rabbit–Alexa 594 (Invitrogen). Adjacent tissue sections were processed with primary antibodies omitted (secondaries only). Images were captured on a Zeiss confocal microscope using a ×63 objective lens.

Flow cytometry. PyMT+/FVB female mice with palpable tumors had injections of PBS or GM-CSF into mammary pad number 4 or 9. After 4 wk, mice were euthanized and their tumors were removed. Immune cells were separated from tumor by centrifugation over a ficoll density gradient. The buffy layer was removed and resuspended in cold fluorescence-activated cell sorting buffer. One half of the cells was incubated 5 min on ice with rat IgG, and then stained with PE anti-mouse FLT-1 or isotype. The other half was permeabilized with the BD Cytofix/Cytoperm kit, incubated 5 min with rat IgG, then stained with the same antibodies. Both sets of samples were fixed with 4% paraformaldehyde, then run on a BD FACScalibur flow cytometer. Analysis was performed using DeNovo FCS Express v3.

Tumor extracellular fluid analyses. Snap-frozen tumor samples from mice treated with PBS (9 samples) or GM-CSF (14 samples) were crushed into fragments <2 mm in diameter and resuspended with 1:2 weight/volume of 2 × PBS and rotated at 4 °C for 2 h. The samples were vortexed then spun for 3 min at 5,000 RPM. The fluid was subjected to cytokine analysis using the Bio-Rad Bioplex for interleukin (IL)-4, IL-10, M-CSF, and VEGF.

Statistical analyses. For data comparing single independent measurements between two treatment groups, Mann-Whitney U test was used to determine whether observations could have come from identical distributions (metastases, tissue necrosis, immunohistochemical stains). The similar nonparametric Kruskal-Wallis test was used when comparing more than two groups (Texas red angiogenesis data). When groups were larger and distributions approximated the normal distribution, equality of means between groups was compared by Student’s t test. Growth data and ongoing oximetry data were compared by repeated measures ANOVA with Tukey post hoc testing because measures of an individual mouse from one observation to the next were not independent. Kaplan-Meier survival analyses used Mantel-Cox Log Rank testing to determine differences between groups.

Figure 1. Intratumor GM-CSF injection slows tumor growth and prolongs survival. A, FVB mice were injected with PyMT tumor cells into the left abdominal mammary fat pad. Upon palpable tumor formation, mice were treated with PBS or 100 ng GM-CSF by intratumor injection thrice per week until tumors reached the maximum size allowed by our Institutional Laboratory Animal Care and Use Committee protocol. Points, mean tumor size from 8 (GM-CSF) and 10 (PBS) mice; bars, SE. P value of 0.025 by repeated measures ANOVA. B, Kaplan-Meier analysis of mice shown in A. Censored data (one mouse in each group) represent mice that died from causes other than tumor burden. Median survival was 3 wks for vehicle-treated mice and 6 wks for GM-CSF–treated mice. Survival in these studies is defined as mice having tumors with a diameter <2 cm, which was criteria for euthanasia in the protocol approved by our IAUC. P value of 0.010 by Mantel-Cox nonparametric log-rank test.
Results

Local treatment with GM-CSF slows tumor growth in a mouse model of breast cancer. Based on our previous work (19), we predicted treating tumors locally with GM-CSF would stimulate TAMs to secrete sVEGFR-1, countering tumor-derived VEGF, and blocking angiogenesis (29). To test this hypothesis, we orthotopically injected MET-1 tumor cells (derived from a PyMT+ FVB mouse) to a single mammary gland of an FVB recipient. We chose to use the PyMT model because (a) tumor progression in this well-characterized model resembles that seen in human tumors (30), (b) these tumors readily metastasize, and (c) the orthotopic allograft injection model maintains a fully functional immune system in these mice. Furthermore, we could isolate the tumor site, minimizing problems associated with tumors arising in 10 separate mammary pads. When tumors became palpable, we randomized the mice into groups. Mice received intratumor injections of equal volumes of PBS or rmGM-CSF thrice per week for 3 wk. Once a week, tumor measurements were recorded.

Mice treated with GM-CSF showed a 66% reduction in tumor size after 3 weeks ($P = 0.025$; Fig. 1A). We let tumors grow until they reached 2 cm in their greatest dimension, at which time the mice were euthanized. Figure 1B shows a 3-week increase in median survival (defined as time to 2-cm tumor diameter) of mice treated with GM-CSF (3 versus 6 weeks; $P = 0.010$). GM-CSF–treated mice had no significant changes in body weight or overt clinical side effects. CBC analysis revealed no difference between treatment groups in the concentrations of any circulating cell type (Total WBCs, neutrophils, lymphocytes, monocytes, eosinophils, basophils, platelets; Mann-Whitney $U, n = 7$ mice/group). In blood collected from mice treated with GM-CSF, serum levels of the cytokine remained undetectable from 6 to 12 hours after intratumor injection (<14 pg/mL; 9 mice per group).

To evaluate the role of inflammation as a variable in the orthotopic injection model, we also injected palpable tumors in native PyMT+ mice with PBS or GM-CSF and compared tumor growth. GM-CSF similarly reduced tumor growth in PyMT+ murine breast cancers, whereas PBS injections did not (data not shown).

**GM-CSF treatment correlates with reduced lung metastasis.** PyMT breast cancers metastasize to the lung (30). Because tumor angiogenesis is essential for metastases and GM-CSF stimulates macrophages to produce sVEGFR-1 (19), we predicted that GM-CSF treatment reduced lung metastases. We orthotopically injected allograft tumors into FVB mice and treated them with GM-CSF or PBS. To enhance lung metastases, all tumors were allowed to grow to 2 cm in diameter before the mice were removed from the study. The lungs of these mice were insufflated, fixed whole, and stained with hematoxylin to identify tumor masses within the lungs (Fig. 2A). GM-CSF treatment reduced the number of lung metastases compared with PBS ($P = 0.037$ by Mann-Whitney $U, 6$ mice per group; Fig. 2B).

**GM-CSF lowers oxygen levels within the tumor proper.** If reduction in tumor growth and metastasis in GM-CSF–treated mice resulted from sVEGFR-1 production and reduced angiogenesis, we predicted these mice would have reduced tumor oxygen levels. To track oxygen concentrations within the tumors of PBS- or GM-CSF–treated mice, we incubated an oxygen-sensitive imaging system showing particle distribution and oxygen tension throughout the tumor. Nanoparticles were uniformly distributed and limited in space to an area corresponding to the tumor proper. Numerical values on oxygen maps represent oxygen concentration in mmHg. $B$, trends in tumor oxygen levels over time. Lines, average oxygen concentration in mmHg taken from two-dimensional EPR measurements ± SE from 5 mice per group. $P$ value of 0.050 by repeated measures ANOVA.

Figure 2. Local GM-CSF treatment reduces tumor metastases to lung. $A$, representative images of lungs from GM-CSF and PBS-treated mice. Arrows, metastatic tumor growths, which appear as small opacities. $B$, quantification of tumor metastases. Dotted line, mean values over 5 mice (PBS) and 6 mice (GM-CSF) per group. $P$ value of 0.037 by Mann-Whitney $U$ test.

Figure 3. Intratumor GM-CSF treatment reduces oxygen levels within mammary tumors in vivo. $A$, representative samples of three-dimensional EPR imaging showing particle distribution and oxygen tension throughout the tumor. Nanoparticles were uniformly distributed and limited in space to an area corresponding to the tumor proper. Numerical values on oxygen maps represent oxygen concentration in mmHg. $B$, trends in tumor oxygen levels over time. Lines, average oxygen concentration in mmHg taken from two-dimensional EPR measurements ± SE from 5 mice per group. $P$ value of 0.050 by repeated measures ANOVA.
nanoprobe (LiNC-BuO microcrystals; ref. 31) with PyMT tumor cells until the probe was internalized and injected these cells into the mammary fat pads of normal FVB female mice. High resolution, three-dimensional EPR techniques analyzed oxygen tension within the tumors. GM-CSF–treated tumors showed reduced oxygen levels compared with tumors treated with PBS ($P = 0.050$ by repeated measures ANOVA; Fig. 3). Distribution maps showed uniform probe allocation throughout the tumor, equal between groups, and limited in space to the tumor itself.

**GM-CSF causes increased cell death within the tumor and changes in patterns of necrosis.** Depriving tumors of oxygen and other nutrients causes cell death and tissue necrosis. To evaluate effects of oxygen deprivation on the tumors, we used fluorescent microscopy to assess the extent of cell death and necrosis in H&E-stained tumor sections (32). Histologic evaluation showed GM-CSF–treated tumors had more tissue necrosis (reflected by enhanced fluorescence) compared with those treated with PBS ($P = 0.011$ by Mann-Whitney $U$; Fig. A4). All tumors analyzed for necrosis were from mice with tumors of 2 cm diameter. Of particular note are the patterns of necrosis. Mice treated with PBS had focal patterns of necrosis with a single area of involvement near the tumor’s outer surface. In contrast, GM-CSF–treated tumors revealed diffuse, multifocal patterns of necrosis, with smaller necrotic foci distributed throughout the tumor.

**GM-CSF increases macrophage but not neutrophil numbers in tumors.** Many different cell types compose murine breast tumors. To examine whether GM-CSF treatment altered the cell types infiltrating the tumor, we analyzed cell influx. We found increased F4/80+ (macrophage) staining within GM-CSF–treated tumors ($P = 0.014$; Fig. 4B) but found no differences in tumor neutrophils (7/4+ cells; 3.18% versus 4.49% 7/4+ cells, 7 and 9 mice per group, $P = 0.315$ by Mann-Whitney $U$).

**Figure 4.** Histologic analysis of tumors. A, H&E sections were imaged at ×10 under green fluorescence conditions. Under these conditions, necrotic tissue will autofluoresce allowing for identification of necrotic regions (left and middle). Necrotic regions were quantified (right) and compared with the area of the entire tumor section to obtain the percent necrotic tissue. $P$ value of 0.011 by Mann-Whitney $U$. B, IHC for F4/80 (brown stain) shows number and distribution of macrophages in tumors treated with vehicle or GM-CSF (left and middle). GM-CSF–treated tumors contain accumulations of F4/80-positive cells around the necrotic areas. Macrophage numbers were digitally quantified by comparing the number of F4/80-positive pixels to the total number of pixels in each high power field for stitched images taken across entire tumors (right). $P$ value of 0.014 by Mann-Whitney $U$. C, ISH identified cells expressing mRNA for sVEGFR-1 (dark blue–black-stained regions). sVEGFR-1–positive cell distribution and phenotype correlate well with macrophage phenotype and distribution, suggesting that these are the cells producing the sVEGFR-1. D, M1/M2 phenotyping assays. Left, immunofluorescence assays assessing M1 (INOS, red) and M2 (arginase-1, green) macrophage polarity. Treatment with GM-CSF caused a shift in these same macrophages identified in C from an M2 phenotype (green) to transitional (yellow) and M1 (red) phenotypes. ECF taken from 9 and 14 tumors per group was subjected to multiplex cytokine bead arrays for IL-4 and IL-10. The results shown in the graphs at the right indicate a significant decrease in tumor levels of these two cytokines that drive M2 differentiation. $P$ values of 0.007 and 0.047 by independent samples $t$ test.
TAMs produce sVEGFR-1 in response to GM-CSF treatment. Because GM-CSF–treated macrophages produce and release sVEGFR-1 (19), we hypothesized that GM-CSF induced TAMs to produce sVEGFR-1. We stained serial tumor sections for F4/80 expression and performed in situ hybridization for sVEGFR-1 mRNA expression. Grossly, GM-CSF– and vehicle-treated tumors looked different. Vehicle-treated tumors had uniform appearance, contrasting with irregular tissue variations seen in the GM-CSF–treated tumors. Not only did GM-CSF increase TAMs but promoted inflammatory nodules, containing mostly F4/80+ macrophages. Macrophages in the GM-CSF–treated tumors produced sVEGFR-1 mRNA, especially along the interface between the nodules and the tumor cells (Fig. 4C). PBS-treated tumors did not develop inflammatory nodules, or express high levels of sVEGFR-1 mRNA.

Administration of GM-CSF causes a shift from M2 to M1 polarization in TAMs. Tumor-educated macrophages are usually M2 phenotype. To test effects of GM-CSF on macrophage polarization, we stained tumor sections for arginase I (M2 macrophages) and iNOS (M1 macrophages). Figure 4D shows that nearly all macrophages from PBS-treated tumors had an M2 phenotype (arginase I, green). In contrast, macrophages from GM-CSF–treated tumors exhibited M1 (iNOS, red) or transitional (double stain, yellow) phenotype macrophages. The highest concentrations of M1 macrophages were found near the inflammatory nodules that surrounded the necrotic tissues.

To assess whether GM-CSF administration elicited a shift in macrophage phenotype within the tumor environment, we extracted extracellular fluid (ECF) from snap-frozen tumor...
samples and measured the M2 cytokines IL-10 and IL-4. Both of these M2 cytokines were significantly lower in tumors treated with GM-CSF.

Identification of sVEGFR-1–producing cells. To provide evidence that GM-CSF stimulated sVEGFR-1 expression in macrophages, we used flow cytometry to assess cells producing this factor. To distinguish between increased membrane-bound versus soluble VEGFR-1, we stained cells isolated from GM-CSF– or vehicle-treated tumors for both surface VEGFR-1 expression and total expression (including intracellular proteins). These studies (Fig. 5) show that the relative numbers of CD68+ cells that express surface-bound VEGFR-1 did not change with GM-CSF treatment. Visual inspection of the plots suggests the emergence of a CD68+ population of cells that did not express surface VEGFR-1. In contrast, treatment with GM-CSF caused a significant increase in a well-defined, CD68HI population of cells, all of which express VEGFR-1.

sVEGFR expression mediates the effects of GM-CSF treatment. We suspected that reduced tumor growth rate was dependent on sVEGFR-1 activity. To test this hypothesis, we injected PyMT tumor cells into the mammary fat pad of normal FVB mice. Upon formation of a palpable tumor, the mice were randomized to one of the following four treatment groups: PBS, GM-CSF, GM-CSF plus a neutralizing antibody to sVEGFR-1, or GM-CSF plus an equimolar concentration of isotype IgG control antibody.

Treatment with a neutralizing antibody to sVEGFR-1 returned the growth rate of GM-CSF–treated tumors to near vehicle-treated levels (P = 0.006 overall, P = 0.046 for GM-CSF + αsVEGFR versus GM-CSF + isotype by repeated measures ANOVA with Tukey post hoc testing; Fig. 6A). In contrast, growth rates for GM-CSF–treated tumors and GM-CSF plus isotype–treated tumors were not different (P = 0.843). To ensure the results we observed were specific for physiologic alteration of the soluble VEGFR-1, and not confounded by binding of the antibody to the cell surface receptors, we also compared growth rates of tumors treated with PBS or anti–sVEGFR-1 alone. There were no differences between these groups (P = 0.923; n = 5 mice per group; data not shown).

Figure 6. sVEGFR neutralization restores normal tumor growth patterns. A, graph shows change in tumor volume over time. Lines, mean tumor size ± SE for each group at each time point. Groups consisted of 4, 5, 8, and 4 mice, respectively. P value of 0.006 overall, P value of 0.046 for GM-CSF + αsVEGFR versus GM-CSF + isotype by repeated measures ANOVA with Tukey post hoc testing. B, mice were injected with a fluorescently labeled dextran immediately before euthanasia. Harvested and sectioned tumors were then imaged with a fluorescent microscope to assess the extent of functional blood vessel development within the tumor. C, representative samples from tumors taken from each group. Stitched images taken across the entire tumor section were digitally quantified for number of fluorescent pixels for each mouse. The average percent of Texas red–positive pixels per mouse is shown in the graph. P value of 0.023 by Kruskal-Wallis test. D, quantification of detectable VEGF levels in ECF. ECF was extracted from 9 PBS-treated and 14 GM-CSF–treated tumors and subjected to multiplex analysis for VEGF. VEGF detection is reduced when bound by sVEGFR-1. Columns, mean detected VEGF concentration; bars, SD. P value of 0.038 by independent samples t test.
GM-CSF treatment reduces vessel density within the tumor. According to our hypothesis, the lower oxygen levels observed in GM-CSF–treated tumors resulted from a sVEGFR-1–mediated block in angiogenesis. To test the effects of GM-CSF on blood vessel growth within the tumor, we injected mice with Texas red–conjugated dextran (70 kDa) 5 minutes before euthanasia. Tumors were removed, fixed, sectioned, and analyzed by fluorescent microscopy. As shown in Fig. 6B to C, GM-CSF treatment reduced the number of vessels in the tumor (P = 0.0235 by Kruskal-Wallis).

GM-CSF treatment reduces detectable levels of VEGF in the tumor. Finally, when we extracted ECF from the tumors and subjected these samples to multiplex cytokine analysis for VEGF, we found a significant reduction in VEGF detection in the GM-CSF–treated tumors (P = 0.038; Fig. 6D). Because the ELISA antibody binds an epitope blocked by sVEGFR-1 (19), this finding is consistent with reduced VEGF activity in the treated samples from the actions of sVEGFR-1.

Discussion

This manuscript presents the novel observation that TAMs can be re-educated in murine breast cancer to an antiangiogenic, antitumor growth and M1 phenotype by local treatment of the tumor with GM-CSF. Understanding the mechanisms of "tumor education" and basic macrophage biology allowed us to identify factors that alter macrophage behaviors within the tumor environment. Our data show that GM-CSF can re-educate TAMs to an antitumor phenotypes in a murine model of breast cancer through the production of sVEGFR-1.

GM-CSF treatment slowed tumor growth and prevented lung metastasis in our mouse model. This approach contrasts with previous work that focused on the strategy of limiting mononuclear phagocyte trafficking to the tumor to reduce tumor growth (15). Our data show that reducing macrophage trafficking to murine breast cancers is not required for therapeutic benefit, but that re-educating the TAMs produced nearly identical effects. In our orthotopic studies, we find increased numbers of TAMs in tumors treated with GM-CSF, and still show improved outcomes. The effects of GM-CSF are robust, especially in light of the aggressive nature of the PyMT tumor model (30). To address the possibility of the orthotopic injection inducing a local inflammatory response that may have contributed to the antitumor effects of GM-CSF, we performed the same studies on PyMT+ transgenic mice. We treated a single mammary tumor with GM-CSF or PBS following the same schedule as our orthotopic allograft model and observed similar results (data not shown).

Because our model for lung metastases required both GM-CSF– and PBS-treated tumors to grow to the same size before sacrifice, lowered numbers of lung metastases suggest that GM-CSF affected the tumor microenvironment (these PyMT tumors do not express GM-CSF receptors). We are actively investigating if GM-CSF treatment limited liberation of tumor cells from the primary tumor or had alternate effects on other factors like reducing tumor intravasation. These questions are being investigated in our laboratory.

In the orthotopic studies, we took advantage of in vivo, intratumor oxygen measurement by EPR as a surrogate for angiogenic activity. Most rapidly growing tumors have large oxygen demands. This demand must be met by the production of new blood vessels to deliver nutrients and oxygen. We found reduced oxygen levels within the tumors of GM-CSF–treated mice, correlating with reduced angiogenesis. This evidence supports the hypothesis that the production of sVEGFR-1 by TAMs blocks functional vessel development. The effects of treatment are especially noticeable in the three-dimensional imaging, where effects at the border of the tumor can be separated from measurements within the tumor (e.g., tumors in GM-CSF–treated mice have <5 mmHg O₂ across ~90% of the tumor by three-dimensional measures at week 3).

Altered amounts and patterns of necrosis found in GM-CSF–treated tumors illustrate the biological significance of oxygen deprivation, and provide outcomes which correlate with reduced angiogenesis and oxygen delivery to the tumors. Differing patterns of necrosis in GM-CSF–treated versus vehicle-treated tumors correlate with macrophage distribution and behavior in the focal nodules of inflammatory cell accumulation throughout the tumor (see discussion of IHC and ISH below).

Reduced levels of functional angiogenesis at 3 weeks confirm that the mechanism behind the GM-CSF effect includes angiogenic blockade. Patterns of blood vessel growth in treated tumors seem more like normalized or physiologic vasculature (33), as opposed to the highly irregular, tortuous patterns of growth seen in untreated tumors or in mice treated with neutralizing antibodies to sVEGFR-1. These patterns correlate well with other studies examining the biological effects of VEGF blockade in patients treated with bevacizumab (34).

We propose that this antiangiogenic activity is attributable to a change in TAM phenotype within the tumor, marked by the expression of sVEGFR-1. Until now, most tumors (especially those of the breast) show strong negative correlation between macrophage infiltration and outcome (6, 35, 36). In the current studies, we reduced rates of breast cancer growth and metastasis despite increasing the number of macrophages in the GM-CSF–treated tumors. We conclude from these data that we have successfully re-educated the TAMs within these tumors—manipulating their behavior to affect desirable outcomes.

Macrophages in these areas also showed a phenotype switch from M2 polarization, typically associated with TAMs to an antitumor, M1 phenotype. Cytokines responsible for driving macrophages toward the M2 phenotype, which is typically associated with TAMs, were also greatly reduced by GM-CSF treatment.

Neutralization of sVEGFR-1 with blocking antibodies shows definitive evidence, that this important antiangiogenic factor largely mediates the effects of GM-CSF treatment on tumor growth. However, sVEGFR-1 blockade did not completely reverse the effects of GM-CSF treatment. Plausible explanations for this include (a) that the blocking antibody had <100% activity or (b) that other mechanisms can account for part of the GM-CSF effect. Others have explored the use of autologous tumor cells engineered to produce GM-CSF to treat human cancers, especially melanoma (37). Their studies show that GM-CSF can stimulate the differentiation and activity of dendritic cells, activating the adaptive immune response to the tumor. These T-cell responses could also occur in the present model. Such effects likely account for at least a portion of the response not due to sVEGFR-1.

Taken together, our data show that GM-CSF reprogrammed TAMs to slow tumor growth by starving the tumors of oxygen and nutrients via increasing levels of sVEGFR-1 within the tumor, preventing angiogenesis. Such models provide a powerful platform for studying the involvement of macrophages and other cells in the processes that comprise malignant transformation. Detailed
studies of the changes invoked by treatment within each cell type and within the tumor organ will help us understand the contributions made by each of the players within this complex environment to the process of malignant transformation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Acknowledgments

Received 4/15/2008; revised 11/17/2008; accepted 12/9/2008; published OnlineFirst 02/17/2009.

Grant support: NIH (RO1HL63800, RO1HL67176, and PO1HL70294-01) and the Department of Defense Congressionally Directed Medical Research Program (BCRP 2005 BC050083).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We would like to thank Dr. Anna Bratzas for her help with the EPR imaging.
Granulocyte Macrophage Colony-Stimulating Factor Inhibits Breast Cancer Growth and Metastasis by Invoking an Anti-Angiogenic Program in Tumor-Educated Macrophages

Tim D. Eubank, Ryan D. Roberts, Mahmood Khan, et al.

Cancer Res 2009;69:2133-2140. Published OnlineFirst February 17, 2009.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-08-1405

Cited articles
This article cites 37 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/69/5/2133.full#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/69/5/2133.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.