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Highly Metastatic 13762NF Rat Mammary Adenocarcinoma Cell Clones Stimulate Bone Marrow by Secretion of Granulocyte-Macrophage Colony-Stimulating Factor/Interleukin-3 Activity

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Circulating neutrophil (polymorphonuclear leukocyte levels rise 50-fold in 13762NF tumor-bearing rats in proportion to the tumor's metastatic potential. Purified tumor-elicited neutrophils enhance metastasis of syngeneic tumor cells when co-injected intravenously; however, circulating and phorbol ester-activated polymorphonuclear neutrophils do not. The purpose of this study was to elucidate the source of tumor-elicited neutrophils in metastatic tumor-bearing rats. We examined the bone marrow in rats bearing tumors of poorly, moderately, and highly metastatic cell clones. Marrow from rats with highly metastatic tumors had increased cellularity (100%), myeloid to erythroid ratio (10:1), and megakaryocytes compared with control rats (cellularity, ~80%; myeloid to erythroid ratio, 5:1), with marrows from rats with moderately metastatic tumors having intermediate values. This suggested production of a colony-stimulating factor by the metastatic cells. To confirm this, bone marrow colony formation from control and tumor-bearing rats was compared. Colony number increased in proportion to the metastatic potential of the tumor. Conditioned medium from metastatic cells supported growth of the granulocyte-macrophage colony-stimulating factor/interleukin-3-dependent 32Dcl3 cell line, but media from nonmetastatic or moderately metastatic cells did not. Antibodies to murine granulocyte-macrophage colony-stimulating factor neutralized 32Dcl3 growth in tumor cell conditioned medium. These results suggest production of a granulocyte-macrophage colony-stimulating factor or interleukin-3-like activity by highly metastatic 13762NF clones and implicate a possible role for colony-stimulating factors in regulating the metastatic potential of mammary adenocarcinoma cell clones. (Am J Pathol 1995, 147:1668–1681)

Metastasis is the spread of cells from a primary tumor to nearby or distant sites. It remains the major reason for cancer deaths. Localized tumors can often be treated effectively, whereas the presence of metastases often means a poor prognosis. To metastasize, a cancer cell must invade the vasculature, survive in the circulation, bind to vascular endothelium, degrade and traverse basement membranes, and grow in a different site.[1–5] Throughout this process, circulating cancer cells are exposed to a variety of insults, including shear forces, antibodies and other serum factors, and components of the immune system.

Although the immune system acts to eliminate tumor cells, metastatic cells and activated leukocytes share many properties, including the ability to attach to endothelium[6,7] as well as degrade and penetrate basement membranes.[8,9] This suggests that under certain circumstances tumor cells might exploit normal leukocyte function to increase the tumor cells' metastatic efficiency.[10,11] The role of the immune system in metastasis has focused on lym-

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phocytes,[12] natural killer (NK) cells,[13–16] and monocytes/macrophages.[12,17,18] Yet neutrophils (also known as polymorphonuclear leukocytes (PMNs) or polys), which make up 45 to 70% of circulating leukocytes in humans could also play an important role.[19,20]

PMNs can be spontaneously cytotoxic to tumor cells[21–24]; and cytotoxicity can be drug enhanced.[22,25–27] Moreover, PMNs have roles other than cytolysis. For example, PMNs can increase attachment to endothelial monolayers,[28] suggesting that PMNs may aid metastasis at the steps of attachment and extravasation. This concept is substantiated by Crissman et al[29] who, in a detailed electron micrograph study, observed circulating PMNs in close association with Lewis lung carcinoma and B16a amelanotic melanoma cells throughout the processes of arrest and extravasation.

We previously showed that rats injected with 13762NF rat mammary adenocarcinoma cell clones developed neutrophilia proportional to the metastatic potential of the primary tumor.[11] Tumor-elicited neutrophils (tcPMNs) increased metastatic potential and invasiveness 2- to 25-fold when co-injected intravenously,[30] whereas normal circulating neutrophils (cPMNs), proteose peptone-elicited neutrophils (pp-PMNs) and phorbol ester-activated neutrophils (TPA-PMNs) could not increase metastasis.[30] Thus, at least two events are occurring in metastatic 13762NF tumor-bearing rats: recruitment of PMNs to the circulation and activation of neutrophils to assist tumor cell invasion and metastasis. The purpose of this study was to determine the mechanism(s) by which metastatic tumor cells elicit neutrophilia.

Two possibilities exist for the origin of tcPMNs: recruitment from marginated pools or stimulation of granulocyte precursors in the bone marrow by direct or indirect stimulation by tumor cells. Leukocytosis,[31] granulocytosis,[32,33] eosinophilia,[31] and neutrophilia[34] have been described in patients with advanced neoplasms of multiple histological types. As in rats with 13762NF tumors, these responses could not be explained by infection or tumor necrosis.[11] In experimental models, the evidence predominantly supports secretion of factors that stimulate bone marrow precursor cells. The CE murine mammary carcinoma causes neutrophilia *in vivo* and conditioned media from cell cultures stimulates neutrophilic colony formation *in vitro*.[34–36] A human pancreatic carcinoma cell line (MIA PaCa-2) produced two distinct colony-stimulating factors in serum-free medium.[37] Likewise, human lung tumor OTUK in nude mice produced a three- to fourfold increase in colony-forming units from femurs isolated from tumor-bearing animals.[38] The factor(s) produced by tumor cells that elicit bone marrow proliferation vary by tumor type, stage, and size.[31,35,38] Lee and colleagues have shown that granulocyte-macrophage colony-stimulating factor (GM-CSF) levels may be correlated with more advanced mammary tumors.[34,35] Granulocyte (G-CSF) and/or GM-CSF mRNAs have been detected in astroglial tumors by reverse transcriptase polymerase chain reaction.[39] Others have found GM-CSF protein or mRNA in tumor cells or effusions by radioimmunoassay, enzyme-linked immunosorbent assay, Northern blot, Western blot, or colony formation assays.[31,37,40,41]

However, the role of colony-stimulating factors like GM-CSF in tumor biology has not been determined. Takeda et al[42] suggest that there may be a correlation of GM-CSF mRNA expression and spontaneous metastasis. Seven of fourteen metastatic transplantable tumors produced GM-CSF mRNA and/or detectable GM-CSF activity, whereas the nonmetastatic tumors did not.[42] In other studies, GM-CSF induced motility of human A2058 melanoma and Lewis lung carcinoma cells.[43,44] Taken together, these data suggest that GM-CSF may play a role in tumor progression and/or metastasis.

Using histology, immunohistochemistry and *in vitro* bone marrow stem cell assays, we report that the source of tcPMNs in 13762NF tumor-bearing rats is bone marrow, not recruitment from marginated stores. Analysis of 13762NF mammary adenocarcinoma cell clone conditioned media revealed that the cells secrete a GM-CSF/interleukin (IL)-3-like activity in proportion to the cells' metastatic ability that is presumably responsible for the bone marrow stimulation. In addition, increased levels of GM-CSF mRNA were noted in highly metastatic clones by Northern blot analysis. Thus, it appears that the highly metastatic clones produce increased levels of GM-CSF, which induces neutrophilia (tcPMNs), which in turn enhances metastatic potential.

Materials and Methods

Tumor Cell Lines and Tissue Culture

13762NF mammary adenocarcinoma clones MTC, MTF7, MTLn2, and MTLn3 were isolated and maintained as described.[45,46] Clones MTC and MTF7 were isolated from a local tumor resulting from implantation of 13762NF mammary adenocarcinoma into the mammary fat pad. MTC is poorly metastatic, whereas MTF7 possesses moderate metastatic potential. MTLn2 and MTLn3, isolated from spontaneous lung metastases, exhibit low and high metas-

tatic potential, respectively. MTLn3(T44).5 and MTLn3(T44).11 were subclones isolated from MTLn3 at passage 44 in culture.[47,48] These subclones have high and low metastatic potentials, respectively.[47] In the 13762NF mammary adenocarcinoma system, rank order of experimental (intravenous (i.v.) inoculation) and spontaneous (mammary fat pad (mfp) inoculation) metastatic potentials are equivalent.[46,47]

Cells were grown in α-modified minimal essential medium (AMEM; Irvine Scientific, Santa Ana, CA) supplemented with 5% fetal bovine serum (Biocell, Carson, CA) but no antibiotics (cAMEM). All cells were maintained in a 37°C humidified atmosphere containing 5% CO_2 in air. Cultures were routinely grown on Corning tissue culture plastic (Corning, Oneonta, NY) and subcultured at a split ratio of 1:50 (MTC, MTF7) to 1:150 (MTLn3, MTLn2) when the cells became approximately 80% confluent, using a 0.25% trypsin solution in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (CMF-DPBS; GIBCO, Grand Island, NY). Cells were screened and found to be free of *Mycoplasma* contamination using the TaKaRa PCR test (Panvera Corp., Madison, WI). The cell lines were also checked (by Microbiological Associates, Bethesda, MD) and found to be free of Sendai, MHV, PVM, Reo3, ectromelia, MVM, polyoma, lactate dehydrogenase, and lymphatic choriomeningitis virus contamination.

As 13762NF mammary adenocarcinoma cells can be phenotypically unstable upon prolonged passage in tissue culture,[46,47] passage numbers were recorded for each experiment. All experiments were internally controlled so that the impact of phenotypic drift on interpretation of results would be minimized. MTC cells were used between tissue culture passages T13 and T25, MTF7 cells were used between passages T25 and T35, MTLn2 cells were used between T38 and T59, and MTLn3 cells were used at high passage (T44 to T65).

GM-CSF-dependent 32Dcl3 cells[49–51] were a gift from Drs. Maribeth Raines (University of California, Los Angeles) and Joel Greenberger (University of Pittsburgh). Cells were passaged in Dulbecco's modified minimal essential medium mixed 1:1 with Ham's F-12 media conditioned by a 1:1000 dilution of media conditioned by COS cells that had been transfected with a murine GM-CSF cDNA in the pXMT2 expression vector[52] using DEAE dextran.[53] The pXMT2-MUCSF expression vector was a gift from the Genetics Institute (Andover, MA).

Conditioned medium from 13762NF cells was prepared by incubating 80% confluent cells with cDME-F12 for 24 hours. The medium was aspirated and centrifuged at 3000 rpm to remove debris and cells.

Monoclonal antibodies that specifically neutralize murine IL-3 or GM-CSF activity were purchased from R&D Systems (Minneapolis, MN). Each antibody produced a dose-dependent growth inhibition of 32Dcl3 cells grown in recombinant murine IL-3- or rmGM-CSF-supplemented medium. Near-complete suppression occurred at >0.05 μg/ml (αIL-3) or >0.5 μg/ml (αGM-CSF). Monoclonal antibodies were preincubated with culture medium before adding cells. Cell counts were done 2 and 4 days after seeding 1×10^5 cells into 24-well tissue culture plates. The ability of the antibodies to neutralize the effects of IL-3 or GM-CSF secreted into the medium by MTLn3 cells was tested similarly.

Animals

Six- to nine-week-old virus- and pathogen-free Fischer 344/NHsd (F344) rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and maintained under specific pathogen-free conditions following the guidelines of the National Institutes of Health and The Pennsylvania State University. Rats were fed (Purina rodent chow) and given water (<5 ppm chlorine) *ad libitum*. Sentinel animals were found to be negative for opportunistic viral and bacterial infections. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Tumor Growth and Experimental Metastasis

Tumor cells were grown to 70 to 80% confluence and harvested as described above. Upon detachment, cells were resuspended in cAMEM to inactivate trypsin. After low speed centrifugation, the cells were resuspended in ice-cold AMEM and counted on a hemacytometer. Cell suspensions were kept on ice at all times and injections were done immediately into the lateral tail veins of non-anesthetized rats for i.v. injections or left inguinal mfp of Metofane-anesthetized rats. Animals were killed by inhalation of Metofane as above when moribund (mfp injections) or after 21 days (i.v. injections) and subjected to complete gross necropsy. The lungs were removed and fixed in 10% neutral buffered formalin:Bouin's fixative (5:1, v/v). Surface pulmonary metastases were counted as described.[46]

Neutrophil Isolation and Purification

Rat neutrophils were isolated from citrate anticoagulated rat blood collected by cardiac puncture with a

21-gauge needle and 10-cc syringe after Metofane anesthesia as described.[11,30] Briefly, anticoagulated blood was diluted with an equal volume of CMF-DPBS and 10 ml overlaid onto 3-ml Ficoll-Paque (Pharmacia, Uppsala Sweden). Centrifugation was at 1900 rpm in a Sorvall RT 6000D table top centrifuge at room temperature for 25 minutes. The cell pellet was resuspended in red blood cell lysis buffer (0.15 mol/L NH_4Cl, 10 mmol/L $KHCO_3$, 0.1 mmol/L disodium EDTA, pH 7.4) and centrifuged at 1200 rpm for 5 minutes. PMNs were resuspended in CMF-DPBS, counted on a hemocytometer, and adjusted to the desired concentration. The PMNs were >99% pure as determined by modified Wright-stained (Leukostat, Fisher Scientific, Pittsburgh, PA) microscopic smears.

tcPMNs were isolated from tumor-bearing rats (2×10^5 cells in 0.2 ml of CMF-DPBS injected into the mfp and incubated ~30 days), and cPMNs were collected from normal rats. TPA-PMN were prepared from cPMN by incubation with 10 ng/ml phorbol myristate acetate (TPA) at 37°C for 1 hour in AMEM without serum.

Histology

Sterna were removed from control and tumor-bearing rats at ~30 days incubation, fixed in 10% neutral buffered formalin for 48 hours, decalcified until sectioning was possible (~2 days), embedded in paraffin, sectioned, and stained with routine Harris hematoxylin and eosin (H&E). Peripheral smears were air dried and stained with a modified Wright's stain.

Immunohistochemistry

Paraffin sections were cut at 5 μm onto silane-coated slides and heated to 80°C for 15 minutes. The tissue was deparaffinized in xylene and ethanol and rehydrated in PBS with 0.1% Triton X-100. Endogenous peroxidase was blocked with methanol/hydrogen peroxide and the slides were rinsed. Rabbit anti-human myeloperoxidase (Dako, Carpinteria, CA) was added at a 1:500 dilution and incubated for 30 minutes. Signet's universal immunoperoxidase rabbit peroxidase anti-peroxidase kit was then used according to the manufacturer's instructions. The entire procedure was carried out on a Fisher histomatic slide stainer, code-on series.

Colony Formation Assay

The colony formation assay was performed in methylcellulose culture media for murine cells from Stem-Cell Technologies (Vancouver, B.C., Canada) according to manufacturer's instructions. Briefly, bone marrow was isolated from rat femurs, dissociated, and purified. Cells were counted, resuspended, and seeded (2×10^6 cells in 1 ml) into methylcellulose in 35-mm petri dishes, then incubated in a humidified atmosphere for 14 to 21 days. All colonies on the dish were then counted under phase-contrast microscopy. Parallel assays were set up in complete (contains growth factors and colony-stimulating factors) and base media (without growth factors) for positive and negative controls, respectively. Marrow was isolated from both control and tumor-bearing rats.

Northern Blots

Poly(A)-enriched RNA (5 μg) was size fractionated in a 1% agarose/formaldehyde gel and blotted onto an Amersham nylon membrane by downward transfer in 20× standard saline citrate (SSC). The RNA was irreversibly bound to the membrane by UV cross-linking. The membrane was prehybridized and then probed with the 1.15-kb *Pst*I fragment of mGM-CSF cDNA at 68°C using the quick hybridization procedure and solutions from Stratagene (La Jolla, CA). The blot was washed at room temperature with 2× SSC/0.1% sodium dodecyl sulfate (SDS), then 0.5% SSC/0.1% SDS, and finally 0.1× SSC/0.1% SDS. The same blot was stripped and reprobed with the 1.3-kb *Pst*I fragment of rat glyceraldehyde phosphate dehydrogenase (GAPDH) as a control for constitutive gene expression.

DNA fragments used for probes were digested and separated on a 0.8% low melting point agarose gel, excised and prepared with PCR Prep (Promega, Madison, WI), labeled by random priming with alpha [^{32}P]dCTP (Amersham). GM-CSF expression was detected by autoradiography for 9 days and GAPDH expression detected after 2 days of exposure. The blot was also exposed to a phosphoroimager screen for 22 hours to directly quantify the signal using a Molecular Dynamics Model 425–120 phosphoroimager (Sunnyvale, CA).

Results

Histology

Histological sections of sternal bone marrow were examined from control, non-tumor-bearing rats as well as rats bearing 13762NF tumors in the mfp after 25 days of growth. The marrow from the control rats showed an overall cellularity of 80% with a myeloid-

to-erythroid (M:E) ratio of 5:1 and numerous megakaryocytes (Figure 1, panel 1, A and B.). Animals with highly metastatic MTLn3 growing in the inguinal mfp showed marked stimulation of the marrow with 100% cellularity and a skewing of the M:E ratio to approximately 10:1. In addition, there was an apparent increase in the absolute number of megakaryocytes (Figure 1, panel 2, A and B). The poorly metastatic MTLn2 produced a marrow nearly identical to the control rats for cellularity, M:E ratio, and numbers of megakaryocyte (Figure 1, panel 1, C and D). The bone marrow of rats bearing the moderately metastatic MTF7 had a cellularity of approximately 90 to 95%, a mild increase in the M:E ratio to approximately 7:1, but minimal to no significant increase in megakaryocytes (Figure 1, panel 1, E and F).

The bone marrow was also examined from rats with poorly and highly metastatic subclones derived from MTLn3. Rats with the poorly metastatic MTLn3(T44).11 show only a mild increase in cellularity (85%) as compared with controls with a mild increase in M:E ratio (Figure 1, panel 2, C and D). However, bone marrow from very highly metastatic MTLn3(T44).5 tumor-bearing rats showed 100% cellularity, with a 10:1 M:E ratio and increased megakaryocytes (Figure 1, panel 2, E and F). Thus, the degree of bone marrow stimulation, as manifest by increased cellularity, M:E ratio, and absolute numbers of megakaryocytes, was proportional to the metastatic potential of the tumor growing in the inguinal mfp in all cases.

Immunohistochemistry

Bone marrow sections were stained by the immunoperoxidase method for the myeloperoxidase enzyme to highlight the alteration in the M:E ratio in tumor-bearing rats and to show that the increase in marrow cellularity was not due to infiltration by metastatic mammary adenocarcinoma cells. A section of human control bone marrow (Figure 2, panel 1A.) was stained as a positive control and a section of rat marrow from MTLn3-bearing rats was stained with nonimmune serum (Figure 2, panel 1B) as a negative control. There was appropriate staining of myeloid cells in the positive control slide with a clean background on the negative control slides. The bone marrow from control, non-tumor-bearing rats showed positive staining of the myeloid cells showing a M:E ratio of approximately 5:1 and indicating cross-reactivity of the anti-human myeloperoxidase for the rat enzyme (Figure 2, panel 1C). A similar pattern is seen in the myeloperoxidase staining of the marrow from rats bearing the poorly metastatic MTLn2 (Figure 2, panel 1D).

The marrow from rats bearing the highly metastatic MTLn3 and MTLn3(T44).5 tumors showed 100% cellularity with positive immunohistochemical staining of most (excluding megakaryocytes and erythroid precursor) cells (Figure 2, panel 2, C and D.) indicating a marked expansion of the myeloid line in this proliferating marrow. Some staining is weak, likely a result of the use of anti-human myeloperoxidase on rat tissues. As adenocarcinoma cells do not express myeloperoxidase, infiltration of the marrow by metastatic tumor is excluded. Rats with the moderately metastatic MTF7 tumor show an intermediate increase in myeloperoxidase positive cells (Figure 2, panel 2A) between the control and MTLn3-stimulated marrow. The poorly metastatic MTLn3(T44).11 tumors produced a marrow with myeloperoxidase staining (Figure 2, panel 2B) similar to that seen in the MTLn2 clones or the controls. These immunoperoxidase findings confirm the H&E data showing an increase in the myeloid lines within the marrow in proportion to the metastatic potential of the growing tumor.

Colony Formation Assay

To further characterize the cellular expansion that was proportional to a tumor's metastatic potential, *in vitro* colony formation assays were performed from control (ie, no tumor), MTLn3-, MTF7-, and MTC-bearing rats (Figure 3). The assay was performed in both media containing growth factors and media without added growth factors as positive and negative controls, respectively. For rats without tumors, an average of 43 colonies formed in each plate. There was a 4.5-fold and 3-fold increase of colony formation from rats with the highly metastatic MTLn3 and moderately metastatic MTF7 tumors, respectively. There was minimal (1.5-fold) increase in colony production for rats with the MTC clone growing in the mfp. Marrow stimulation is proportional to the metastatic potential of the growing tumor, as measured by *in vitro* colony formation.

32Dcl3 Growth Curves as a Bioassay for GM-CSF/IL-3 Activity

The GM-CSF-dependent 32Dcl3 cells were grown in media containing various dilutions of GM-CSF (Figure 4A). In the absence of GM-CSF, the cells senesce and die over a period of 3 to 5 days. The growth rate of these cells is proportional to the con-

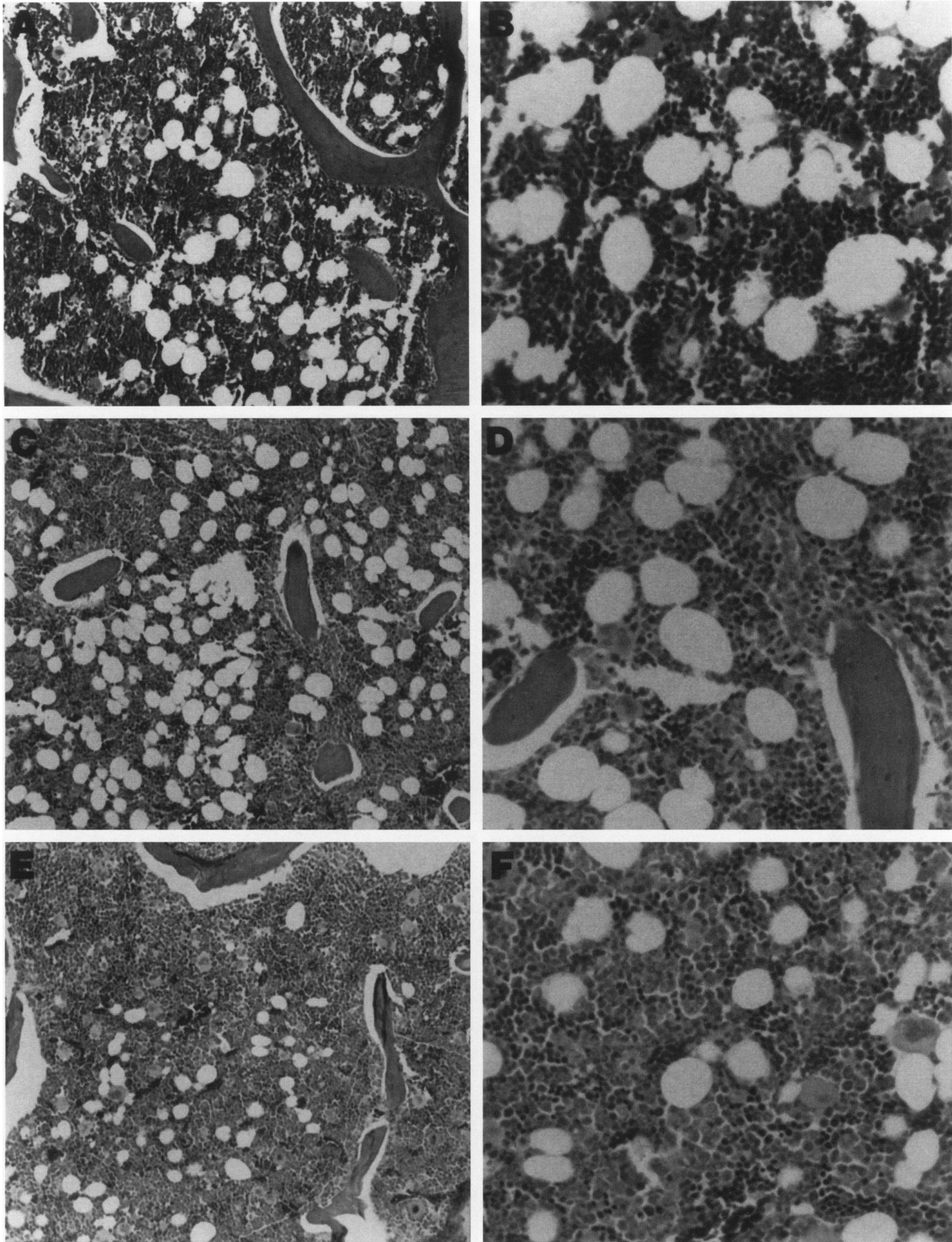
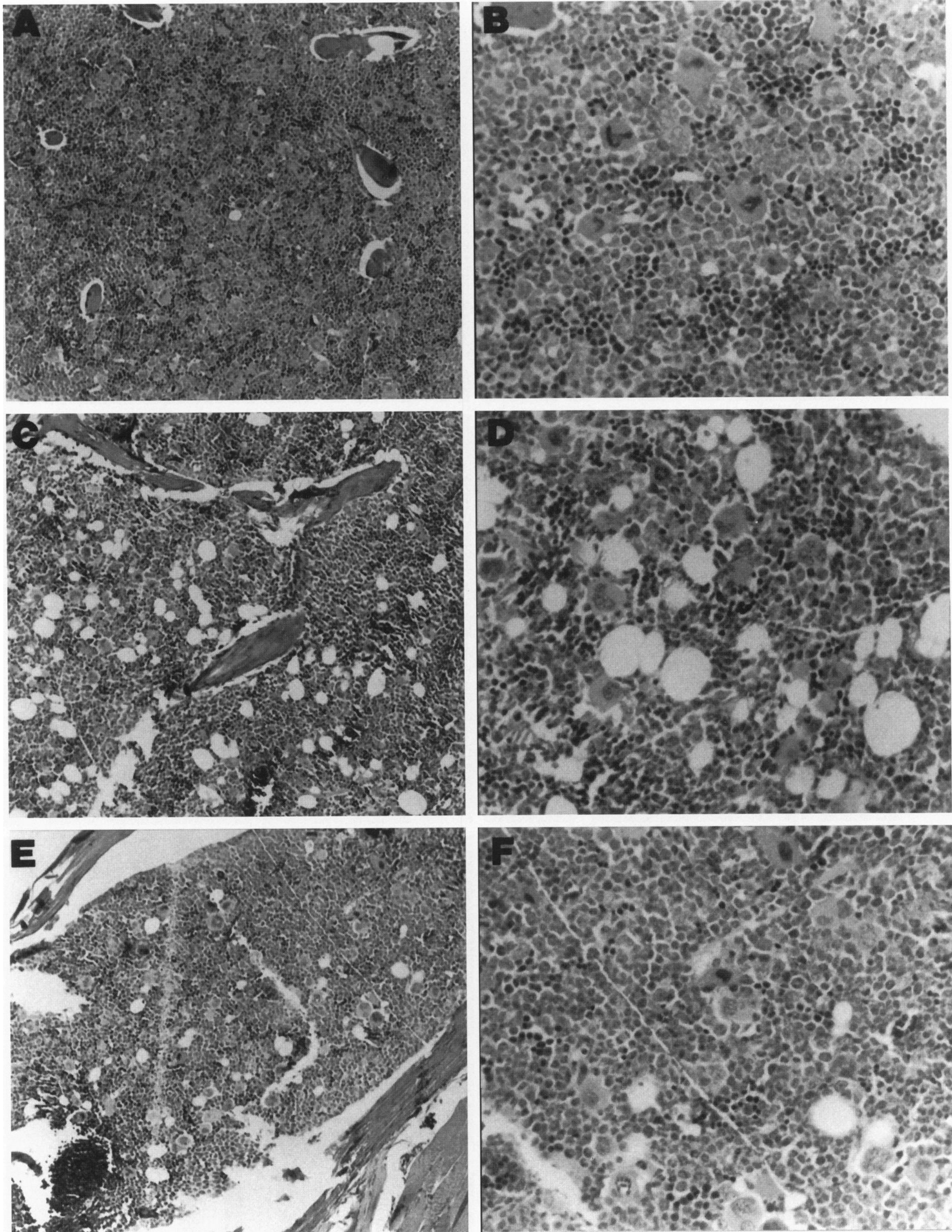


Figure 1. *Rat sternal bone marrow histology. A representative field is shown at a magnification of ×63 and ×160. Identical results were seen on sections from four separate control and tumor-bearing rats.* Panel 1, A *and* B: *Control non-tumor-bearing rats;* C *and* D: *MTLn2-bearing rats;* E *and* F: *MTF7-bearing rats.* Panel 2, A *and* B: *MTLn3-bearing rats;* C *and* D: *MTLn3 (T44).11-bearing rats;* E *and* F: *MTLn3 (T44).5-bearing rats.*



centration of GM-CSF within the culture media with dilutions ranging from 1:2 to 1:64 from a stock solution obtained from COS cells transfected with mGM-CSF. Although the precise amount of GM-CSF in the COS-conditioned media was not known, there is clear concentration dependence.

To better characterize the bioassay, a growth curve was performed with media containing known

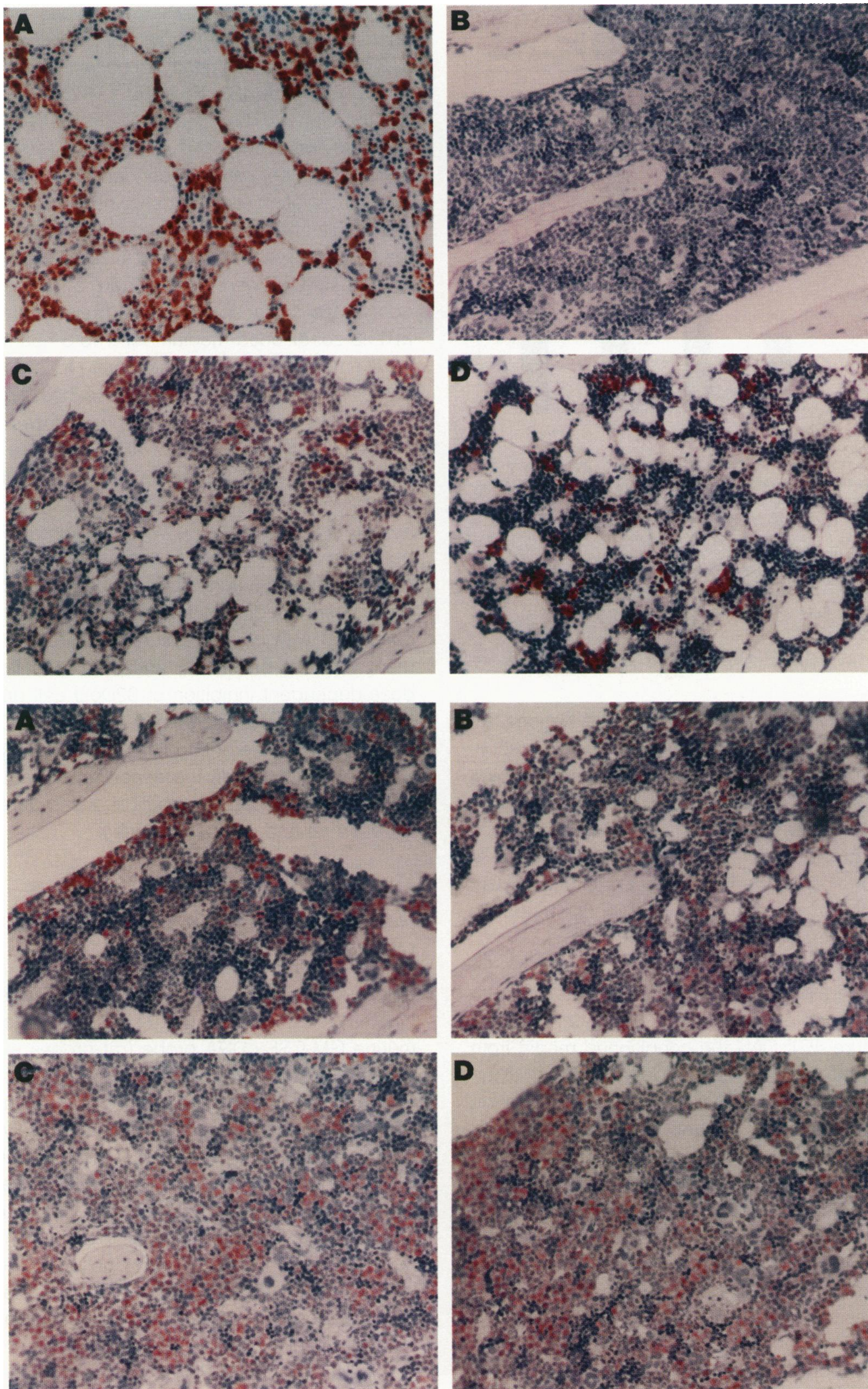


Figure 2. *Myeloperoxidase immunohistochemistry of rat sternal bone marrow.* Panel 1A: *Human marrow positive control;* B: *Nonimmune antibody stain, MTLn-3 tumor-bearing rat, negative control;* C: *normal rat bone marrow;* D: *MTLn2-bearing rats.* Panel 2A: *MTF7-bearing rats;* B: *MTLn3 (T44).11-bearing rats;* C: *MTLn3-bearing rats;* D: *MTLn3 (T44).5-bearing rats.*

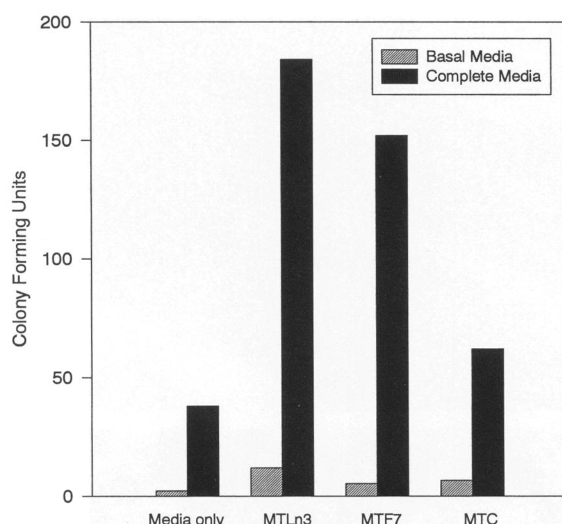


Figure 3. *Bone marrow colony formation assay from control and 13762NF rat mammary adenocarcinoma tumor-bearing rats. Syngeneic rats (four per group) were injected in the inguinal mfp with 1 × 10^6 cells of the indicated 13762NF cell clone. Tumors formed and, at 25 to 30 days, the animals were killed, femoral bone marrow was collected in heparinized media, and the assay was performed as described in Materials and Methods. Control non-tumor-bearing, MTLn3-bearing, MTF7-bearing, and MTC-bearing rats were assayed. Identical results were obtained on two separate experiments.*

amounts of GM-CSF obtained from the National Institute for Biological Standards and Control. rmGM-CSF was obtained in 100,000-U ampules containing 1 μg of GM-CSF in 0.9% NaCl, 0.1% trehalose, and 0.2% human serum albumin. GM-CSF (500 U/ml) yields a growth rate equivalent to the COS-transfected conditioned media at full strength (Figure 4B). In addition, growth rate was GM-CSF concentration dependent in the range of 5 to 500 U/ml.

13762NF mammary adenocarcinoma clone conditioned media was then examined for its ability to support growth of the GM-CSF-dependent 32Dcl3 cells (Figure 4C). Only the conditioned media from the highly metastatic MTLn3 supported growth of the 32Dcl3 cells to any significant extent. Media conditioned by MTC and MTF7 cells supported only minimal growth (Figure 4C). Comparing the growth rate of the 32Dcl3 cells in MTLn3-conditioned media with the rate obtained in 500 U/ml standard GM-CSF allowed calculation of the amount of GM-CSF-like activity secreted by the adenocarcinoma clones. MTLn3 cells secrete >>50 U/ml GM-CSF/IL-3 activity whereas MTC and MTF7 secrete <10 U/ml activity. In some experiments, MTLn3 cells were calculated to produce >2000 U/ml.

Because the 32Dcl3 cells we obtained were derived from an IL-3-dependent line, we tested the cells for a response to standard preparations of murine IL-3 (in 0.9% NaCl, 0.1% trehalose, and 0.2% human serum albumin). Growth of the 32Dcl3 cells in IL-3 (Figure 4D.) was nearly identical to the growth in GM-CSF (Figure 4B). Thus the 13762NF adenocarcinoma clone MTLn3 secretes an activity with GM-CSF/IL-3-like activity. MTLn3-secreted GM-CSF/IL-3 activity is concentration dependent and is diluted out at a 1:10 dilution (data not shown).

To discriminate between IL-3 and GM-CSF effects, neutralizing monoclonal antibodies specific to IL-3 or GM-CSF were used (Figure 5). Varying concentrations of antibody were preincubated with MTLn3-conditioned medium for 1 to 3 hours before addition of 32Dcl3 cells. Anti-murine IL-3 antibodies completely inhibited growth of 32Dcl3 cells grown in the presence of rmIL-3 (50 U/ml) at doses greater than 0.05 μg/ml but did not alter growth in rmGM-CSF-containing medium (data not shown). Likewise, anti-mGM-CSF inhibited growth at doses >0.5 μg/ml in medium supplemented with 50 U/ml rmGM-CSF but did not affect growth in IL-3-containing medium. Addition of anti-murine IL-3 antibodies to MTLn3-conditioned medium inhibited growth 20 to 40% compared with control cultures (Figure 5A). Treatment with anti-mGM-CSF antibodies resulted in a dose-dependent inhibition of 32Dcl3 cell growth in MTLn3-conditioned medium, with $IC_{50} \approx 1.0$ μg/ml (Figure 5B). As the antibodies are species specific, decreased potency in the conditioned medium experiments is probably a result of subtle differences between rat and murine cytokines.

Northern Blot Analysis of IL-3 and GM-CSF mRNAs

Poly(A)-enriched RNA from the 13762NF adenocarcinoma clones were analyzed by Northern blot with a murine GM-CSF cDNA probe (rat probes were unavailable). The GM-CSF mRNA was of very low abundance, producing only faint bands on the autoradiogram (not shown). Blots were also analyzed by phosphoroimager (Table 1). Highly metastatic MTLn3 and MTLn3(T44).5 clones yielded a much stronger signal (up to eightfold) than did the moderately or poorly metastatic 13762NF clones when normalized for GAPDH expression. The level of GM-CSF mRNA is proportional to the metastatic potential of the clone and to the degree of neutrophilia and bone marrow stimulation observed.

A poly(A)-enriched RNA blot (5 μg/lane) was analyzed with multiple probes recognizing several regions of murine IL-3 (designer probes, R&D Systems). IL-3 mRNA was also of low abundance,

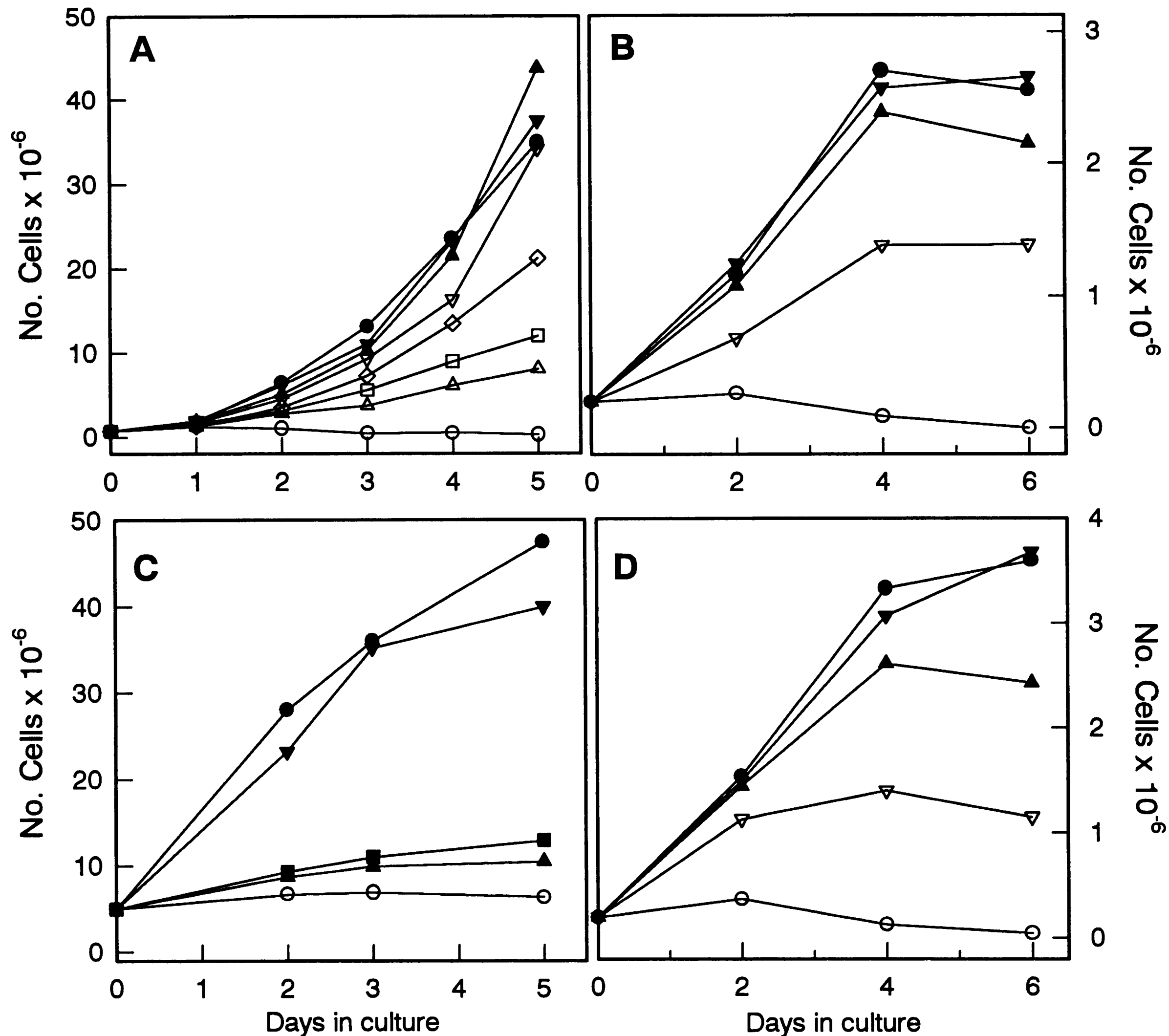


Figure 4. *32Dcl3 cell bioassay for GM-CSF/IL-3 activity. 32Dcl3 cells were seeded onto 24-well plates at 1 × 10^5 to 5 × 10^5 cells/well in growth medium.* A: *Serial dilutions of conditioned media from GM-CSF-transfected COS cells: undiluted (●), 1:2 (▼), 1:4 (▲), 1:8 (▽), 1:16 (◇), 1:32 (□), 1:64 (△), negative control (○).* B: *GM-CSF (prepared from samples provided by the National Institute for Biological Standards and Control) added at concentrations of 500 (▼), 50 (▲), and 5 (▽) U/ml.* C: *13762NF cell clone conditioned medium: MTLn3 (▼), MTF7 (▲), MTC (■).* D: *IL-3 (prepared from samples provided by the National Institute for Biological Standards and Control) at 500 (▼), 50 (▲), and 5 (▽) U/ml. For all* panels*: positive control (●, GM-CSF-transfected COS-cell-conditioned medium) and negative control (○, basal medium). Symbols represent the mean. Standard errors bars were generally smaller than the symbols and were not included for ease of viewing the data.*

producing recognizable bands after 3 days of exposure. The IL-3 band intensity was quantified by densitometry and normalized to GAPDH levels to correct for loading differences (Table 2). Highly metastatic MTLn3 and MTLn3(T44).5 clones expressed more IL-3 mRNA (up to 7.5-fold) than less metastatic MTC, MTF7, or MTLn2 cells. IL-3 expression in the low to moderately metastatic subclone MTLn3(T44).11 was approximately equal to that of highly metastatic MTLn3. Thus, although less metastatic cells generally transcribe less IL-3 than the more metastatic clones, the correlation is imperfect.

Discussion

Tumors are composed of a mixture of multiple subsets of cells that differ for many phenotypes, including metastatic potential. Some tumor cells capitalize on host responses to enhance malignant behavior. For example, several types of tumor cells promote platelet aggregation to increase embolus size[54,55] and some form heterotypic aggregates with other immune cells (reviewed in Ref. 56). Both of these processes result in increased efficiency of intracapillary arrest of tumor cells. Although PMNs comprise

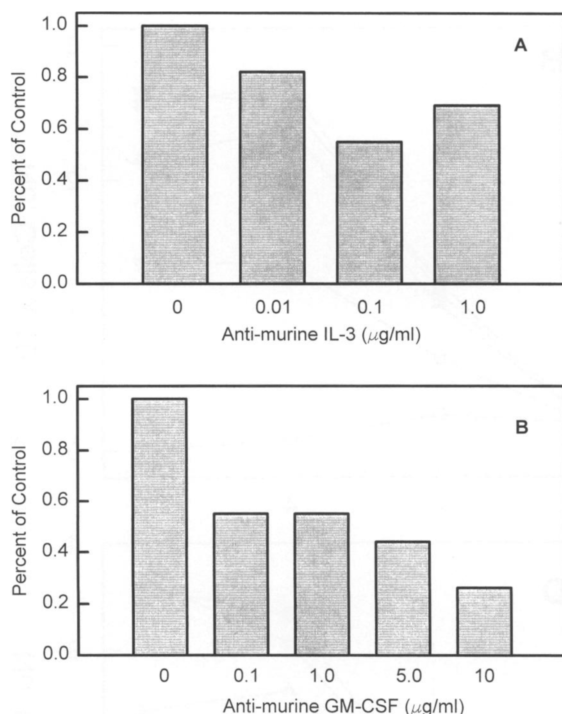


Figure 5. *32Dcl3 cell growth in MTLn3-cell-conditioned medium. 32Dcl3 cells were seeded onto 24-well plates at 1 × 10^5 cells/well after media treated for 1 to 3 hours with neutralizing monoclonal anti-murine IL-3* (**A**) *or anti-murine GM-CSF* (**B**) *antibodies at the concentrations listed. Cell counts were done 3 days after seeding in quadruplicate samples. Error bars are not shown but were less than 2% for replicate cultures.*

the major class of granulocytes in humans and are often involved in the immune response to tumors, little is known regarding their relationship to metastatic potential.

We previously found a marked neutrophilia in 13762NF mammary adenocarcinoma tumor-bearing rats; the level of increase correlated with metastatic potential (r = 0.92). Isolated tcPMNs were able to enhance tumor cell invasion and metastasis.[57] However, the source of the tcPMNs was not known. It could either be recruitment from marginated pools or stimulation of bone marrow granulocyte precursors.

Table 1. *GM-CSF mRNA Expression in 13762NF Adenocarcinoma Clones*

Cell line	Metastatic potential	Arbitrary units (phosphoroimager) GM-CSF	Murine GM-CSF/ GAPDH
MTC	Low	586	337
MTLn2	Low	0	
MTF7	Intermediate	716	213
MTLn3	High	4354	1656
MTLn3(T44).11	Low-moderate	1338	711
MTLn3(T44).5	High	3477	5519

mRNA was separated on a formaldehyde-agarose gel and probed with murine GM-CSF and GAPDH. Quantification was done with a phosphoroimager. Raw counts are shown as well as data normalized to murine GAPDH. The results are consistent with enhanced production of GM-CSF correlating with increasing metastatic potential.

Table 2. *IL-3 mRNA Expression in 13762NF Adenocarcinoma Cells*

Cell line	Metastatic potential	IL-3/GAPDH (arbitrary OD units)
MTC	Low	0.15
MTLn2	Low	0.09
MTF7	Intermediate	0.08
MTLn3	High	0.24
MTLn3(T44).11	Low-moderate	0.27
MTLn3(T44).5	High	0.60

mRNA was separated on a formaldehyde-agarose gel and probed with a mixture of murine IL-3 probes (R&D Systems) and GAPDH. Quantification was done with a densitometer and data normalized to GAPDH. The results are consistent with production of IL-3 by the different cells.

Histological examination of sterna from syngeneic tumor-bearing rats revealed that the cellularity was significantly greater in metastatic tumor-bearing rats than control rats. H&E-stained sections and myeloperoxidase-stained sections confirmed that the marrows displayed a greater cellular component and that the shift of M:E ratios in the rats was proportional to the number of circulating tcPMNs. Thus, it is likely that the tcPMNs resulted from stimulation of bone marrow precursor cells by the tumor cells either directly or indirectly (ie, via an intermediary cell). The types of cells observed in the sections indicated stimulation with GM-CSF or IL-3. Subsequent experiments were performed to determine whether GM-CSF or IL-3 were produced by the tumor cells.

Conditioned media from metastatic 13762NF rat mammary adenocarcinoma cells supported bone marrow proliferation. The ability of conditioned medium to sustain growth of IL-3/GM-CSF-dependent 32Dcl3 cells suggested that 13762NF mammary adenocarcinoma cells secreted GM-CSF or IL-3. Interestingly, poorly metastatic clones were incapable of supporting growth of 32Dcl3 cells, even when conditions controlled for cell number and time for which the medium was conditioned. Taken together, our results strongly suggest that metastatic 13762NF rat mammary adenocarcinoma cells secrete a GM-CSF- or IL-3-like molecule that induces proliferation of granulocyte precursor and megakaryocyte precursor populations in syngeneic bone marrow. This conclusion is further supported by experiments in which neutralizing antibodies to GM-CSF, and to a lesser extent IL-3, abrogate the growth of 32Dcl3 cells in MTLn3 tumor cell conditioned medium. The latter results must be interpreted with caution as antibod-

ies directed against murine cytokine were used to measure neutralization of rat cytokine activity. Failure to interact sufficiently with rat IL-3 may explain the modest effects observed with anti-IL-3 antibody in the growth experiments. Secretion of these cytokines forms the basis for the marked neutrophilia in tumor-bearing rats.

The increased number of megakarocytes in the marrow is somewhat surprising in that an increase in platelet counts was not observed in 13762NF tumor-bearing rats. As platelet aggregation around circulating tumor cells can increase metastatic efficiency,[54,55] it was possible that stimulation of megakaryocyte precursors, with a corresponding increase in platelet count, could increase metastasis. This hypothesis is less likely because the ability of 13762NF rat mammary adenocarcinoma cells to induce platelet aggregation does not correlate with metastasis.[58] However, we cannot rule out the possibility based solely on our data.

As GM-CSF and IL-3 are involved in the stimulation of bone marrow progenitors to produce granulocyte populations in healthy individuals,[59,60] it is not yet clear how the PMNs are further stimulated to assist tumor cell invasion and metastasis while neutrophils from non-tumor-bearing rats cannot. Besides causing bone marrow proliferation, GM-CSF can induce neutrophil activation,[61] motility,[7] and stimulation of tyrosine kinases.[50,62] Therefore, it is possible that GM-CSF performs a dual function: recruitment of PMNs from bone marrow and further activation to assist tumor cell invasion and metastasis.

Besides stimulation of bone marrow, GM-CSF produced by metastatic tumor cells could also function in an autocrine manner as this cytokine has previously been shown to increase tumor cell motility.[43] In addition, GM-CSF is implicated in lung surfactant homeostasis.[63] As the major site for 13762NF metastasis is lungs, GM-CSF may participate in conditioning the "soil" for subsequent colonization. These alternatives will require additional study.

One further implication of these results relates to the clinical use of GM-CSF in the treatment of cancer.[64–66] Although the predominant role in cancer therapy is as an adjuvant (1) to heighten immune responses against the tumor or (2) to shorten the myelosuppression resulting from chemotherapy, our data suggest that GM-CSF may, under some circumstances, actually increase the malignant behavior of some tumor cells. As GM-CSF production by tumor cells is not limited to the 13762NF system, this possibility must be weighed.

In summary, metastatic 13762NF rat mammary adenocarcinoma cells secrete a GM-CSF- or IL-3-like activity that causes proliferation of myelocytic populations of syngeneic bone marrow. This, in turn, results in neutrophilia proportional to the metastatic potential of the primary tumor. These results demonstrate yet another mechanism by which tumor cells subvert normal physiology to spread to distant sites.

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References

1. Price JE: Host-tumor interactions in the progression of breast cancer metastasis. In Vivo 1994, 8:145–154
2. Tarin D, Matsumura Y: Recent advances in the study of tumour invasion and metastasis. J Clin Pathol 1994, 47:385–390
3. Fidler IJ: Critical factors in the biology of human cancer metastasis. Cancer Res 1990, 50:6130–6138
4. Nicolson GL: Growth mechanisms and cancer progression. Hosp Prac 1993, 28:43–53
5. Nicolson GL: Cancer metastasis: tumor cell and host organ properties important in metastasis to secondary sites. Biochim Biophys Acta 1988, 948:175–224
6. Hoover HC, Ketcham AS: Techniques for inhibiting tumor metastasis. Cancer 1975, 35:5–14
7. Yong KL, Linch DC: Granulocyte-macrophage colony-stimulating factor differentially regulates neutrophil migration across IL-1-activated and nonactivated human endothelium. J Immunol 1993, 150:2449–2456
8. Wright DG, Gallin JI: Secretory responses of human neutrophils. J Immunol 1979, 123:285–294
9. Klotz K-N, Jesaitis AJ: Neutrophil chemoattractant receptors and the membrane skeleton. BioEssays 1994, 16:193–198
10. Gorelik E, Wiltrout RH, Brunda MJ, Holden HT, Herberman RB: Augmentation of metastasis formation by thioglycolate-elicited macrophages. Int J Cancer 1982, 29: 575–581
11. Aeed PA, Nakajima M, Welch DR: The role of polymorphonuclear leukocytes (PMN) on the growth and metastatic potential of 13762NF mammary adenocarcinoma cells. Int J Cancer 1988, 42:748–759
12. Kripke ML: Ultraviolet radiation and immunology: something new under the sun: Presidential address. Cancer Res 1994, 54:6102–6105
13. Hanna N: The role of natural killer cells in the control of tumor growth and metastasis. Biochim Biophys Acta 1985, 780:213–226

14. Herberman RB: Possible role of natural killer cells and other effector cells in immune surveillance against cancer. J Invest Dermatol 1984, 83:137–140
15. Garafolo A, Chriivi RGS, Scanziani E, Mayo JG, Vecchi A, Giavazzi R: Comparative study on the metastatic behavior of human tumors in nude, beige/nude/Xid and severe combined immunodeficient mice. Invasion Metastasis 1993, 13:82–91
16. Woodruff MF: The cytolytic and regulatory role of natural killer cells in experimental neoplasia. Biochim Biophys Acta 1986, 865:43–57
17. Mantovani A: Tumor-associated macrophages in neoplastic progression: a paradigm for the *in vivo* function of chemokines. Lab Invest 1994, 71:5–16
18. Key ME: Macrophages in cancer metastasis and their relevance to metastatic growth. Cancer Metastasis Rev 1983, 2:75–88
19. Glaves D: Correlation between circulating cancer cells and incidence of metastasis. Br J Cancer 1983, 48: 665–673
20. Smith JA: Neutrophils, host defense, and inflammation: a double-edged sword. J Leukocyte Biol 1994, 56:672–686
21. Kondo M, Kato H, Yoshikawa T, Sugino S: Treatment of cancer ascites by intraperitoneal administration of a streptococcal preparation of OK-432 with fresh human complement: role of complement-deprived chemotactic factor to neutrophils. Int J Immunopharm 1986, 85: 715–721
22. Barker E, Reisfeld RA: A mechanism for neutrophil-mediated lysis of human neuroblastoma cells. Cancer Res 1993, 53:362–367
23. Pickaver AH, Ratcliffe NA, Williams AE, Smith H: Cytotoxic effects of peritoneal neutrophils on a syngeneic rat tumour. Nature New Biol 1972, 235:186–187
24. Fisher B, Saffer EA: Tumor cell cytotoxicity by granulocytes from peripheral blood of tumor-bearing mice. J Natl Cancer Inst 1978, 60:687–691
25. Cameron DJ: Role of hydrocortisone, chloroquine and prednisolone in neutrophil mediated cytotoxicity. Jpn J Exp Med 1986, 56:207–212
26. Ikename M, Mizuno D, Yamazaki M: Drug-dependent cellular cytotoxicity mediated by polymorphonuclear leukocytes. Jpn J Cancer Res (GANN) 1985, 76:637–643
27. Inoue T, Sendo F: *In vitro* induction of cytotoxic polymorphonuclear leukocytes by supernatant from a concanavalin-A-stimulated spleen cell culture. J Immunol 1983, 131:2508–2514
28. Starkey JR, Liggitt HD, Jones W, Hosick HL: Influence of migratory blood cells on the attachment of tumor cells to vascular endothelium. Int J Cancer 1984, 34: 535–543
29. Crissman JD, Hatfield J, Schaldenbrand M, Sloane BF, Honn KV: Arrest and extravasation of B16 amelanotic melanoma in murine lungs: a light and EM study. Lab Invest 1985, 53:470–478
30. Welch DR, Schissel DJ, Howrey RP, Aeed PA: Tumor-elicited polymorphonuclear cells, in contrast to "normal' circulating polymorphonuclear cells, stimulate invasive and metastatic potentials of rat mammary adenocarcinoma cells. Proc Natl Acad Sci USA 1989, 86:5859–5863
31. Sawyers CL, Golde DW, Quan S, Nimer SD: Production of granulocyte-macrophage colony-stimulating factor in two patients with lung cancer, leukocytosis and eosinophilia. Cancer 1992, 69:1342–1346
32. Suda T, Muira Y, Mizoguchi H, Kubota K, Takaku F: A case of lung cancer associated with granulocytosis and production of colony-stimulating activity by the tumour. Br J Cancer 1980, 41:980–984
33. Hughes WF, Higley CS: Marked leukocytosis resulting from carcinomatosis. Ann Intern Med 1952, 37:1085–1088
34. Lee MY, Liu CC, Lottsfeldt JL, Judkins SA, Howard GA: Production of granulocyte-stimulating and bone-cell-modulating activities from a neutrophilia hypercalcemia-inducing murine mammary cancer cell line. Cancer Res 1987, 47:4059–4065
35. Lee MY, Baylink DJ: Hypercalcemia, excessive bone resorption and neutrophilia in mice bearing a mammary carcinoma. Proc Soc Exp Biol 1983, 172:424–429
36. Lee MY, Lottsfeldt JL: Augmentation of neutrophilic granulocyte progenitors in the bone marrow of mice with tumor-induced neutrophilia: cytochemical study of *in vitro* colonies. Blood 1984, 64:499–506
37. Wu M-C, Cini JK, Yunus AA: Purification of a colony-stimulating factor from cultured pancreatic carcinoma cells. J Biol Chem 1979, 254:6226–6228
38. Asano S, Urabe A, Okabe T, Sato N, Kondo Y, Ueyama Y, Chiba S, Ohsawa N, Kosaka K: Demonstration of granulopoietic factor(s) in the plasma of nude mice transplanted with a human lung cancer and in the tumor tissue. Blood 1977, 49:845–852
39. Nitta T, Sato K, Allegretta M, Brocke S, Lim M, Mitchell DJ, Steinman L: Expression of granulocyte colony stimulating factor and granulocyte-macrophage colony stimulating factor genes in human astrocytoma cell lines in glioma specimens. Brain Res 1992, 571:19–25
40. Fu Y-X, Watson GA, Kasahara M, Lopez DM: The role of tumor-derived cytokines on the immune system of mice bearing a mammary adenocarcinoma. I. Induction of regulatory macrophages in normal mice by the *in vivo* administration of rGM-CSF. J Immunol 1991, 146: 783–789
41. Mano H, Nishida J, Usuki K, Maru Y, Kobayashi Y, Hirai H, Okabe T, Urabe A, Takaku F: Constitutive expression of the granulocyte-macrophage colony-stimulating factor gene in human solid tumors. Jpn J Cancer Res 1987, 78:1041–1043
42. Takeda K, Hatakeyama K, Tsuchiya Y, Rikiishi H, Kumagai K: A correlation between gm-csf gene expression and metastasis in murine tumors. Int J Cancer 1991, 47:413–420
43. Kohn EC, Hollister GH, DiPersio JD, Wahl S, Liotta LA,

Schiffmann E: Granulocyte-macrophage colony-stimulating factor induces human melanoma cell migration. Int J Cancer 1993, 53:968–972

44. Young MRI, Halpin J, Hussain R, Lozano Y, Djordjevic A, Devata S, Matthews JP, Wright MA: Inhibition of tumor production of granulocyte-macrophage colony-stimulating factor by 1α,25-dihydroxyvitamin D_3 reduces tumor motility and metastasis. Invasion Metastasis 1993, 13:169–177
45. Neri A, Welch DR, Kawaguchi T, Nicolson GL: Development and biologic properties of malignant cell sublines and clones of a spontaneously metastasizing rat mammary adenocarcinoma. J Natl Cancer Inst 1982, 68:507–517
46. Welch DR, Neri A, Nicolson GL: Comparison of "spontaneous' and "experimental' metastasis using rat 13762 mammary adenocarcinoma cell clones. Invasion Metastasis 1983, 3:65–80
47. Welch DR, Krizman DB, Nicolson GL: Multiple phenotypic divergence of mammary adenocarcinoma cell clones. I. *In vitro* and *in vivo* properties. Clin Exp Metastasis 1984, 2:333–355
48. Welch DR, Evans DP, Tomasovic SP, Milas L, Nicolson GL: Multiple phenotypic divergence of mammary adenocarcinoma cell clones. II. Sensitivity to radiation, hyperthermia and FUdR. Clin Exp Metastasis 1984, 2:357–371
49. Adams EG, Bhuyan BK: Effects of 7-*R*-*O*-methylnogarol (Menogaril) on L1210 cell progression *in vitro* and *in vivo*. Cancer Res 1986, 46:6125–6130
50. Corey SJ, Burkhardt AL, Bolen JB, Geahlen RL, Tkatch LS, Tweardy DJ: Granulocyte colony-stimulating factor receptor signaling involves the formation of a three-component complex with Lyn and Syk protein-tyrosine kinases. Proc Natl Acad Sci USA 1994, 91:4683–4687
51. Raines MA, Liu L, Quan SG, Joe V, DiPersio JF, Golde DW: Identification and molecular cloning of a soluble human granulocyte-macrophage colony stimulating factor receptor. Proc Natl Acad Sci USA 1991, 88: 8203–8207
52. Miyatake S, Otsuka T, Yokota T, Lee F, Arai K: Structure of the chromosomal gene for granulocyte-macrophage colony-stimulating factor: comparison of the mouse and human genes. EMBO J 1985, 4:2561–2568
53. Ausubel FM, Brent R, Kingston RE, et al: Current Protocols in Molecular Biology. New York, John Wiley & Sons, 1990
54. Gasic G, Gasic TB, Galanti N, Johnson T, Murphy S: Platelet-tumor-cell interactions in mice: the role of platelets in the spread of malignant disease. Int J Cancer 1973, 11:704–718
55. Honn KV, Tang TG, Chen YQ: Platelets and cancer metastasis: more than an epiphenomenon. Semin Thromb Hemost 1992, 18:392–415
56. Nicolson GL: Cancer metastasis: organ colonization and the cell-surface properties of malignant cells. Biochim Biophys Acta 1982, 695:113–176
57. Tomasovic SP, Armour EP, North SM, Welch DR: Rat mammary adenocarcinoma heat-stress proteins *in vivo*. Int J Hyperthermia 1987, 3:467–473
58. Estrada J, Nicolson GL: Tumor cell-platelet aggregation does not correlate with metastatic potential of rat 13762NF mammary adenocarcinoma tumor cell clones. Int J Cancer 1984, 34:101–105
59. Metcalf D: Control of granulocytes and macrophages: molecular, cellular and clinical aspects. Science 1991, 254:529–534
60. Metcalf D: Hemopoietic regulators [Review]. Trends Biochem Sci 1992, 17:286–289
61. Conti P, Reale M, Barbacane RC, Panara MR, Bongrazio M, Fiore S, Mier JW, Dempsey RA: Granulocyte-macrophage colony stimulating factor potentiates human polymorphonuclear leukocyte aggregation responses to formyl-methionyl-leucyl-phenylalanine. Immunol Lett 1992, 32:71–80
62. Berkow RL: Granulocyte-macrophage colony-stimulating factor induces a staurosporine inhibitable tyrosine phosphorylation of unique neutrophil proteins. Blood 1992, 79:2446–2454
63. Dranoff G, Crawford AD, Sadelain M, Ream B, Rashid A, Bronson RT, Dickersin GR, Bachurski CJ, Mark EL, Whitsett JA, Mulligan RC: Involvement of granulocyte-macrophage colony-stimulating factor in pulmonary homeostasis. Science 1994, 264:713–716
64. Quesenberry PJ, Lowry PA: The colony stimulating factors: an overview. Cancer 1992, 70:909–912
65. Demetri GD, Antman KHS: Granulocyte-macrophage colony stimulating factor (GM-CSF): preclinical and clinical investigations. Semin Oncol 1992, 19:362–385
66. Steward WP: Granulocyte and granulocyte-macrophage colony-stimulating factors. Lancet 1993, 342:153–157