Non-Small Cell Lung Cancer Cells Induce Monocytes to Increase Expression of Angiogenic Activity[1]

Eric S. White, Scott R. B. Strom, Noel L. Wys, and Douglas A. Arenberg[2]

Tumors are dependent on angiogenesis for survival and propagation. Accumulated evidence suggests that macrophages are a potentially important source of angiogenic factors in many disease states. However, the role(s) of macrophages in non-small cell lung cancer (NSCLC) have not been determined. We hypothesized that monocyte-derived macrophages are induced by NSCLC to increase expression of angiogenic factors. To define the role of macrophage-tumor cell interaction with respect to angiogenesis, human peripheral blood monocytes (PBM) were cocultured with A549 (human bronchoalveolar cell carcinoma) or Calu 6 (human anaplastic carcinoma) NSCLC cells. The resultant conditioned medium (CM) was evaluated for angiogenic potential and for expression of angiogenic factors. We found that endothelial cell chemotactic activity (as a measure of angiogenic potential) was significantly increased in response to CM from cocultures of PBM/NSCLC compared with PBM alone, NSCLC alone, or a combination of NSCLC and PBM CM generated separately. Subsequent analysis by ELISA reveals markedly increased CXC chemokine expression, with a lesser increase in vascular endothelial growth factor, in CM from PBM/NSCLC coculture. Neutralizing Ab to angiogenic CXC chemokines blocked the increase in endothelial cell chemotaxis. Furthermore, with separately generated CM as a stimulus, we found that macrophages are the predominant source of increased CXC chemokine expression. Finally, we found that NSCLC-derived macrophage migration-inhibitory factor is responsible for the increased expression of macrophage-derived angiogenic activity. These data suggest that the interaction between host macrophages and NSCLC cells synergistically increases angiogenic potential, and that this is due to an increased elaboration of angiogenic CXC chemokines. *The Journal of Immunology,* **2001, 166: 7549–7555.**

To grow $>2-3$ mm^3, tumors must induce angiogenesis to create a new blood supply (1–3). Regulation of tumor angiogenesis occurs through a balance of angiogenic (4–6) and angiostatic factors (3, 7–9). Hanahan and Folkman (3) have suggested that quiescent endothelium exist in a net balance of angiostatic factors over angiogenic ones, thereby maintaining homeostasis. Conversely, in malignancy, there is a predominance of angiogenic factors or a relative decrease in angiostatic factors tipping the balance in favor of neovascularization (6, 9–11). The cell type(s) that affect the balance of angiogenic factors may be the malignant cells (12, 13) or the tumor stroma (14, 15).

Multiple studies have demonstrated the association between increased tumor vascularity and macrophage infiltration (16–19), suggesting that the presence of macrophages enhances the angiogenic potential of tumors. It also has been shown that tumor samples with higher macrophage counts tend to be associated with poorer patient prognosis (19–21). These observations suggest that the presence of macrophages within tumors may promote angiogenic activity. Furthermore, it may be the interaction between tumor cells and macrophages that induces macrophages to produce angiogenic factors.

Because macrophages are potentially important sources of angiogenic factors in cancer, we hypothesized that interaction between non-small cell lung cancer (NSCLC)[3] and peripheral blood monocytes (PBM) would enhance the angiogenic potential of tumors. In this study, we show that although NSCLC and PBM both induce endothelial cell chemotaxis, coculture of PBM with NSCLC leads to a synergistic increase in angiogenic activity that is attributable to increased expression of angiogenic CXC chemokines. Furthermore, we have found that macrophage migration-inhibitory factor (MIF) is, in large part, the NSCLC-derived factor which accounts for the increased macrophage-derived angiogenic activity.

Materials and Methods

Cell lines and reagents

Human lung non-small cell carcinoma cells (A549; ATCC CCL-185; American Type Culture Collection, Manassas, VA) and human PBM were maintained in RPMI 1640, 10% FCS supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1% L-glutamine, and 2.5% HEPES buffer. A second human lung carcinoma cell line (Calu 6; ATCC HTB-56) was maintained in Eagle's minimum essential medium/10% FCS with 0.1 mM nonessential amino acids and 1 mM sodium pyruvate. Human dermal microvascular endothelial cells (HMVEC) (Cell Systems, Kirkland, WA) were maintained per the supplier's recommendations in CS-C complete medium. Human microvascular endothelial cells (HMEC-1) kindly provided by Dr. E. Ades and Mr. F. J. Candal (Center for Disease Control, National Center for Infectious Diseases, Atlanta, GA) and Dr. T. Lawley (Emory University, Atlanta, GA), were maintained in MCDB131 medium

[3] Abbreviations used in this paper: NSCLC, non-small cell lung cancer; PBM, peripheral blood monocyte; MIF, migration-inhibitory factor; HMVEC, human dermal microvascular endothelial cells; HMEC, human microvascular endothelial cells; CM, conditioned medium; ENA, epithelial neutrophil-activating peptide; GRO, growth-related oncogene; IP, IFN-γ-inducible protein; MIG, monokine induced by IFN-γ; VEGF, vascular endothelial growth factor; hpf, high-power field.
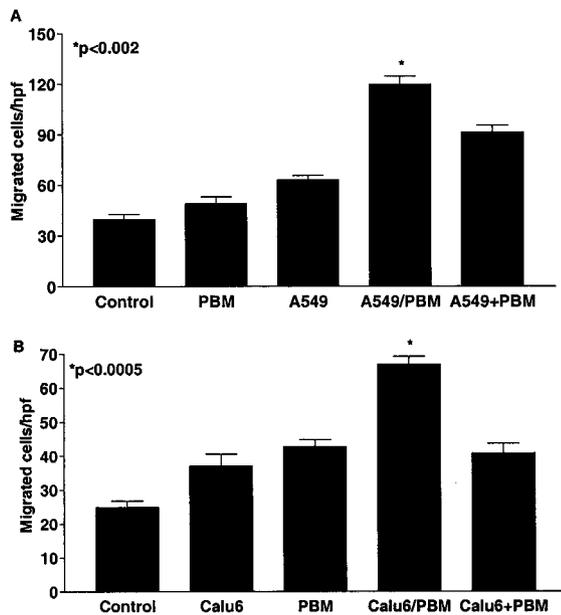
FIGURE 1. Endothelial cell chemotaxis to CM from A549 cells, Calu 6 cells, PBMs, or coculture of A549 or Calu 6 with PBM. Data shown are representative examples of a minimum of three repeated experiments. Results were similar whether HMEC-1 or HMVEC cells were used. *A,* Endothelial cell chemotaxis to CM from A549 cells (63.1 ± 2.6 cells/hpf), PBMs (49.1 ± 4.0), coculture CM (119.9 ± 4.7 cells/hpf), or separately generated A549 and PBM CM (91.4 ± 4.1). ∗, $p < 0.002$ for the comparison of coculture medium to either A549 CM, PBM CM, or separately generated A549 and PBM CM. *B,* Endothelial cell chemotaxis to CM from Calu 6 cells (18.6 ± 2.1 cells/hpf), PBMs (19.5 ± 2.6 cells/hpf), coculture CM (36.7 ± 1.6 cells/hpf), or separately generated Calu 6 and PBM CM (17.3 ± 0.9 cells/hpf). ∗, $p < 0.0005$ for the comparison of coculture CM to either Calu 6 CM, PBM CM, or separately generated Calu 6 and PBM CM.

(Life Technologies, Grand Island, NY) to which FCS (10%), 100 U/ml penicillin, 100 μg/ml streptomycin, 1% L-glutamine, 10 ng/ml epidermal growth factor, and 1 μg/ml hydrocortisone had been added. Medium was changed in all cell lines every 48–72 h. All cell lines were incubated at 37°C with 5% CO_2.

Purified and biotinylated Ab for epithelial-neutrophil activating peptide (ENA)-78, growth-related oncogene (GRO)-α, IFN-γ-inducible protein (IP)-10, monokine induced by IFN-γ (MIG), MIF, and vascular endothelial growth factor (VEGF) were obtained from R&D Systems (Minneapolis, MN). Abs for GRO-γ were obtained from PeproTech (Rocky Hill, NJ). Purified and biotinylated Abs for IL-8 were produced as described previously (22).

Monocyte isolation

After informed consent was obtained, whole blood was obtained by venipuncture from healthy donors and anticoagulated with 1000 U heparin/60 ml blood drawn. Mononuclear cells were isolated by Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation. Remaining red blood cells were lysed by washing with ACK buffer (1 mM $KHCO_3$, 0.15 mM NH_4Cl, and 0.1 mM Na_2EDTA). Resultant mononu-

clear cells (~30% monocytes and 70% lymphocytes) were washed twice with HBSS without Mg^{2+} or Ca^{2+}, and resuspended in serum-free RPMI 1640 with 0.1% BSA at a concentration of 3×10^6 cells/ml. To generate monocyte-conditioned medium (CM), 500 μl of the suspension was plated out in each well of a 24-well plate. Plates were incubated at 37°C with 5% CO_2 for 1 h, and nonadherent cells were removed by washing with RPMI 1640. Wells then were filled with 500 μl of serum-free RPMI 1640, and specimens were incubated for 48 h at 37°C with 5% CO_2. At 48 h, CM was harvested and centrifuged to remove any cell debris. All samples were stored at −20°C until used in assays.

Monocyte/NSCLC coculture

A549 or Calu 6 cells were grown to 80–90% confluence in six-well plates. Cells then were washed twice with serum-free medium, and PBM (isolated as above) were added into the upper chamber of a 25-mm transwell insert (Nunc, Naperville, IL) and allowed to adhere for 1 h. Cells were washed twice with RPMI 1640 and then transwell inserts were placed directly into A549-containing wells. The upper chamber then was filled with 1 ml of serum-free medium. Cocultures were incubated for 48 h at 37°C with 5% CO_2. At 48 h, CM was harvested and centrifuged at $10,000 \times g$ for 5 min. All CM was stored at −20°C until used for the various assays. In some experiments, PBM were exposed to NSCLC CM, and NSCLC cells were exposed to PBM CM.

Endothelial cell chemotaxis assay

Chemotaxis membrane filters (5.0-μm pore size; Osmonics, Livermore, CA) were first prepared by bathing in 2.8% glacial acetic acid for 24 h, followed by two 1-h rinses in deionized, distilled water. The filters then were transferred to a 0.01% gelatin bath and allowed to soak for 24 h. Filters were dried under a laminar flow hood and stored at room temperature for up to 1 month. Endothelial cell chemotaxis assays were performed in 12-well, blind well chemotaxis chambers (Neuroprobe, Cabin John, MD). Briefly, human endothelial cells were suspended at a concentration of 10^6 cells/ml in their respective serum-free medium (in the case of HMEC-1, MCDB-131 medium was supplemented with 0.1% BSA). Endothelial cells were placed into each of the bottom wells (165 μl). Membranes were placed over the wells, the gasket applied, and the chambers sealed. Chambers were inverted and incubated at 37°C with 5% CO_2 for 1 h (HMEC-1) or 2 h (HMVEC) to allow for endothelial cell adherence to the membrane. Chambers then were reinverted and the CM to be studied was placed in the upper well (116 μl). Chambers were reincubated for 2 h. Membranes then were scraped to remove any adherent, unmigrated endothelial cells from the lower chamber, fixed in methanol, stained with a modified Wright-Giemsa stain, and cells that had migrated through the membrane were counted in five high-power fields (hpf; ×200). Results were expressed as the mean number of endothelial cells that had migrated per hpf ± SEM. Each sample was assessed in triplicate. Experiments were performed at least three times. Results obtained were similar whether HMEC-1 or HMVEC cells were used. Each experiment was accompanied by a positive control (VEGF 100 ng/ml) and a negative control (nonconditioned medium) to account for variable chemotaxis from one assay to the next (data not shown).

ELISA

CXC chemokines (IL-8 (CXCL8), ENA-78 (CXCL5), GRO-α (CXCL1), MIG, (CXCL9); and IP-10, (CXCL10)), MIF, and VEGF were quantitated by a double-ligand method described previously (5). In brief, flat-bottom 96-well microtiter plates (Immuno Plate F96; Nunc) were coated with the appropriate purified Abs overnight at 4°C and were then washed three times with PBS with 0.05% Tween 20 (washing buffer). Nonspecific binding sites were blocked with PBS and 2% BSA. After washing three times,

Table I. *ELISA analysis of A549, PBM, Calu 6, or coculture CM for CXC chemokines and VEGF[a]*

Condition	IL-8 (CXCL8)	ENA-78 (CXCL5)	GROα (CXCL1)	IP-10 (CXCL10)	MIG (CXCL9)	VEGF
A549	0.3 ± 0.1	18.1 ± 1.1	0.3 ± 0.01	ND	0.4 ± 0.1	3.7 ± 0.8
Calu 6	0.5 ± 0.2	ND	1.2 ± 0.1	0.11 ± 0.01	0.5 ± 0.1	2.3 ± 0.1
PBM	26.4 ± 6.0	27.5 ± 7.5	8.4 ± 2.6	0.03 ± 0.01	0.6 ± 0.1	ND
A549/PBM	53.7 ± 7.1*	67.8 ± 9.4*	31.8 ± 10.1*	1.2 ± 0.5*	0.9 ± 0.2	2.5 ± 0.12
Calu 6/PBM	44.5 ± 2.9**	67.1 ± 15.9**	48.5 ± 9.6**	2.1 ± 0.5**	0.7 ± 0.1	0.8 ± 0.1

[a] Results are expressed as nanograms per milliliter ± SEM. ND, Levels below the lower limit of detection (<1 pg/ml). ∗, $p \leq 0.01$ for comparison with PBM alone or A549 alone. ∗∗, $p \leq 0.05$ for comparison with PBM alone or Calu 6 alone. Data are representative of three repeated experiments.

samples (neat and 1:10 dilution) and standards were added as 50-μl aliquots and incubated at 37°C. Plates were washed three times and incubated with the respective biotinylated Ab at 37°C. Plates were again washed three times and incubated with polyperoxidase-avidin substrate (Endogen, Woburn, MA) at 37°C. Subsequently, plates were incubated with the peroxidase substrate (Dako, Carpinteria, CA) at room temperature to the desired extinction. The reaction was terminated with 0.5 M H_2SO_4. Plates were read at 490 nm in an ELISA reader. Standards were prepared as ½ log dilutions of purified recombinant Ab, from 100 ng/ml to 0.001 ng/ml per well. For VEGF and MIF, we developed an ELISA by performing multiple assays on standards of human recombinant protein from 1pg/ml to 100ng/ml using varying concentrations of coating and detecting Abs until optimal working concentrations were defined. VEGF ELISA used 20 ng/ml for coating and 75 ng/ml for detecting, and consistently and specifically detected quantities of $VEGF_{121}$ and $VEGF_{165}$ (the predominant secretory forms of human VEGF) at concentrations as low as 1 pg/ml. For MIF, 75 ng/ml was used for coating, and 20ng/ml was used for detection. This ELISA had a similar sensitivity and specificity (data not shown).

Immunodepletion of MIF from NSCLC CM

A total of 250 mg of Protein A immobilized on Sepharose beads (Sigma, St. Louis, MO) was reconstituted in 15 ml of PBS (pH 8.0) overnight at 4°C, washed twice, then brought up in PBS to a final concentration of 150 mg/ml. A total of 1 μg of goat anti-human MIF or goat IgG (R&D Systems) was added to 100 μl of the protein A-Sepharose solution and brought up to a total volume of 500 μl in PBS, mixed thoroughly, and incubated for 2 h at 4°C. The beads then were washed and resuspended in 100 μl of PBS. Then1 ml of A549 or Calu 6 CM was added to the beads and incubated on a rocker plate for 3 h at 4°C. The precipitate then was removed by centrifugation at 7500 $\times$ g for 5 min. Supernatants then were removed and used to stimulate adherence purified PBMs. One aliquot of each CM was subjected to specific ELISA to confirm that MIF had been depleted by the immunoprecipitation.

Statistical analyses

All generated data were compared by Student's t test for unpaired observations, and were considered significant if p values were < 0.05. Results were presented as the mean number of migrated endothelial cells/hpf $\pm$ SEM. All data are representative of a minimum of three separate experiments. Data were analyzed on a Dell computer with the StatView 5.0.1 statistical software package (SAS Institute, Cary, NC).

Results

NSCLC cells and monocytes demonstrate constitutive expression of angiogenic activity that is synergistically increased in PBM/NSCLC coculture

We hypothesized that because monocyte-derived macrophages are found within tumors, these two cell types may interact to increase angiogenic activity. To test this hypothesis, we performed endothelial cell chemotaxis assays on medium generated from the coculture of PBMs and NSCLC cell lines. In comparison to CM from PBM alone, A549 CM alone, or a combination of the two separately generated CM, coculture CM demonstrated a marked increase in angiogenic activity as assessed by endothelial cell chemotaxis ($p < 0.002$; Fig. 1A). Similarly, PBM CM alone, Calu 6 CM alone, or separately generated Calu 6 and PBM CM demonstrated significantly less angiogenic activity than coculture CM ($p < 0.0005$; Fig. 1B).

Coculture of PBM with NSCLC cell lines is associated with increased expression of angiogenic CXC chemokines

To determine the degree to which CXC chemokines or VEGF were responsible for the increased endothelial cell chemotaxis seen in NSCLC and PBM CM, we assessed levels of the angiogenic CXC chemokines IL-8 (CXCL8), ENA-78 (CXCL5), GRO-α (CXCL1), and angiostatic CXC chemokines IP-10 (CXCL10) and MIG (CXCL9) as well as VEGF by ELISA. Coculture of PBM with NSCLC resulted in a marked increase in angiogenic CXC chemokine expression with a much smaller change in VEGF levels. In contrast, expression of angiostatic CXC chemokines remained
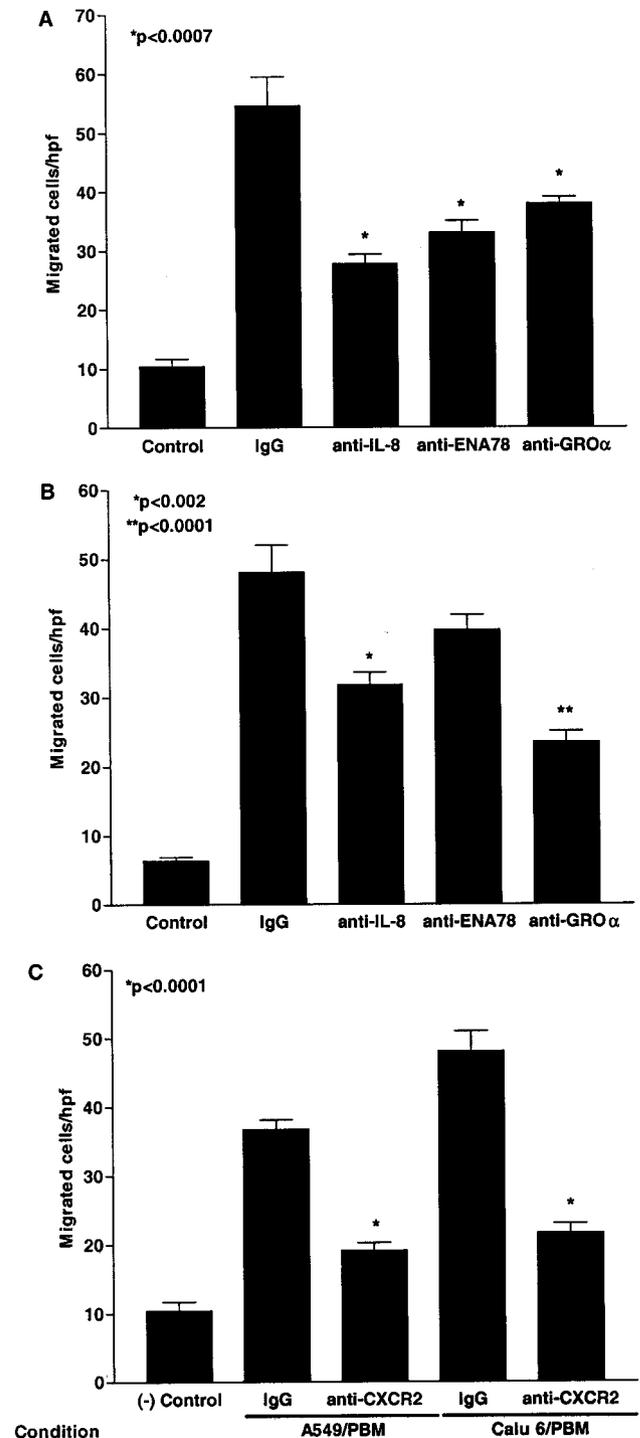

FIGURE 2. *A*, Endothelial cell chemotaxis assay to A549/PBM coculture CM in the presence of neutralizing Abs to IL-8 (CXCL8; 27.7 $\pm$ 1.6 cells/hpf), ENA-78 (CXCL5; 33 $\pm$ 2.0 cells/hpf), GRO-α (CXCL1; 37.9 $\pm$ 1.1 cells/hpf), or control IgG (54.6 $\pm$ 4.9 cells/hpf; $p < 0.0007$ for all conditions). *B*, Endothelial cell chemotaxis to Calu 6/PBM coculture CM in the presence of neutralizing Abs to IL-8 (CXCL8; 31.8 $\pm$ 1.8 cells/hpf), ENA-78 (CXCL5; 39.7 $\pm$ 2.2 cells/hpf), GRO-α (CXCL1; 23.5 $\pm$ 1.6 cells/hpf) or control IgG (48.1 $\pm$ 3.9 cells/hpf; $p < 0.002$ compared with anti-IL-8 (CXCL8); $p = 0.08$ for anti-ENA-78 (CXCL5); $p < 0.0001$ compared with anti-GRO-α (CXCL1)). *C*, Endothelial cell chemotaxis to A549/PBM coculture CM. Endothelial cells were preincubated with either control IgG (48.1 $\pm$ 2.9 cells/hpf) or anti-CXCR2 (21.67 $\pm$ 1.34 cells/hpf; $p < 0.0001$). *D*, Endothelial cell chemotaxis to Calu 6/PBM coculture CM. Endothelial cells were preincubated with either control IgG (36.7 $\pm$ 1.4 cells/hpf) or anti-CXCR2 (19.1 $\pm$ 1.1 cells/hpf; $p < 0.0001$). Data are representative of a minimum of three repeated experiments.

Table II.　*ELISA analysis of CM generated from PBM cultured in NSCLC cell-free CM or NSCLC cells cultured in PBM cell-free CM for CXC chemokines and VEGF*[a]

Condition	IL-8 (CXCL8)	ENA-78 (CXCL5)	GROα (CXCL1)	IP-10 (CXCL10)	MIG (CXCL9)	VEGF
A549/PBM	195.1 ± 27.9	25.1 ± 4.3	194.7 ± 21.1	1.4 ± 0.2	ND	2.5 ± 0.1
A549 in PBM CM	6.6 ± 0.6	3.3 ± 0.2	53.6 ± 3.5	ND	ND	5.9 ± 0.1
PBM in A549 CM	324.8 ± 2.0*	36.0 ± 3.8	294.8 ± 44.7	ND	ND	1.5 ± 0.2
Calu 6/PBM	323.5 ± 58.2	46.9 ± 5.7	103.2 ± 4.2	ND	ND	6.3 ± 0.6
Calu 6 in PBM CM	108.6 ± 2.3	1.6 ± 0.1	1.4 ± 0.4	ND	ND	0.8 ± 0.01
PBM in Calu 6 CM	406.7 ± 49.3	282.6 ± 42.5	124.4 ± 3.9	ND	ND	ND

[a] Levels are expressed as ng/ml of CM ± SEM. Calu 6 in PBM = Calu 6 cells cultured in cell-free PBM CM. PBM in Calu 6 = PBM cultured in cell-free Calu 6 CM. PBM in A549 = PBM cultured in cell-free A549 CM. A549 in PBM = A549 cells cultured in PBM cell free CM. A549/PBM and Calu 6/PBM are coculture CM. Data are representative of three repeated experiments.

very low (Table I). LPS contamination was excluded as a cause of increased CXC chemokine expression by adding polymyxin B to coculture, which had no effect on NSCLC-induced CXC chemokine expression from PBMs. LPS levels were below the limit of detection by *Limulus* lysate assay (data not shown).

Increased endothelial cell chemotaxis in response to coculture CM is markedly reduced in the presence of neutralizing Ab to angiogenic CXC chemokines or their receptor, CXCR2

We hypothesized that the increased endothelial cell chemotaxis to PBM/NSCLC coculture was attributable to the observed increase in angiogenic CXC chemokines. To test this hypothesis, endothelial cell chemotaxis was performed with PBM/NSCLC coculture CM in the presence of neutralizing Ab to IL-8 (CXCL8), ENA-78 (CXCL5), and GRO-α (CXCL1). We found that endothelial cell chemotaxis to A549/PBM coculture CM was significantly reduced in the presence of neutralizing Abs to either IL-8 (CXCL8), ENA-78 (CXCL5), or GRO-α (CXCL1) as compared with control IgG ($p < 0.0007$; Fig. 2*A*). Similarly, endothelial cell chemotaxis to Calu 6/PBM coculture CM in the presence of neutralizing Ab to IL-8 (CXCL8) or GRO-α (CXCL1) was significantly less than in the presence of control IgG ($p < 0.002$; Fig. 2*B*). Recently, CXCR2 was identified as the receptor mediating the angiogenic activity of CXC chemokines (23). Therefore, we performed endothelial cell chemotaxis to coculture CM using endothelial cells preincubated with neutralizing Ab to CXCR2 or control IgG. We found neutralizing Ab to CXCR2 markedly reduced the increased angiogenic response to coculture CM (Calu 6/PBM or A549/PBM; Fig. 2*C*; $p < 0.0001$ for both conditions).

PBM are the primary source of increased angiogenic CXC chemokine expression in PBM/NSCLC coculture CM

We demonstrated that angiogenic CXC chemokine levels are markedly increased when PBM are cocultured with NSCLC. To determine the source of the increased chemokine levels, we first incubated PBM in cell-free NSCLC CM and NSCLC in cell-free PBM CM to assess chemokine expression. We found that when PBM are incubated with CM from NSCLC, there is a significant increase in expression of the angiogenic CXC chemokines similar to that seen when PBM are cocultured with NSCLC cells (Table II). Interestingly, NSCLC cells cultured in cell-free PBM CM also expressed increased levels of angiogenic CXC chemokines as compared with baseline expression (Tables I and II), albeit to a much lesser degree. This suggests that PBM are the predominant source of the increased chemokine expression in coculture. All CM were tested and found to be free of endotoxin by *Limulus* lysate assay (data not shown).

Constitutively expressed, NSCLC-derived, macrophage MIF is responsible for increased expression of angiogenic activity by PBMs

Recently, MIF has been associated with tumor angiogenesis (24–27), but it has not been shown to be directly angiogenic. We hypothesized that MIF may be the tumor-derived factor that induces angiogenic activity from PBMs. To test this hypothesis, we first measured levels of MIF in 48-h CM from unstimulated NSCLC cell lines. MIF was detected in both A549 (4.8 ± 0.2 ng/ml) and Calu 6 CM (6.9 ± 1.0 ng/ml). To determine whether MIF was responsible for increased PBM-derived angiogenic activity, we performed endothelial cell chemotaxis assays on CM from PBMs that had been exposed to Calu 6 or A549 CM. Before stimulation of PBMs, NSCLC CM was subjected to immunodepletion with either IgG or goat anti-human MIF Ab. Depletion of MIF was confirmed by ELISA (data not shown). We found that NSCLC CM depleted of MIF was significantly less effective at inducing PBM-derived angiogenic activity ($p < 0.001$; Fig. 3). Furthermore, PBM stimulated with MIF-depleted A549 or Calu 6 CM resulted
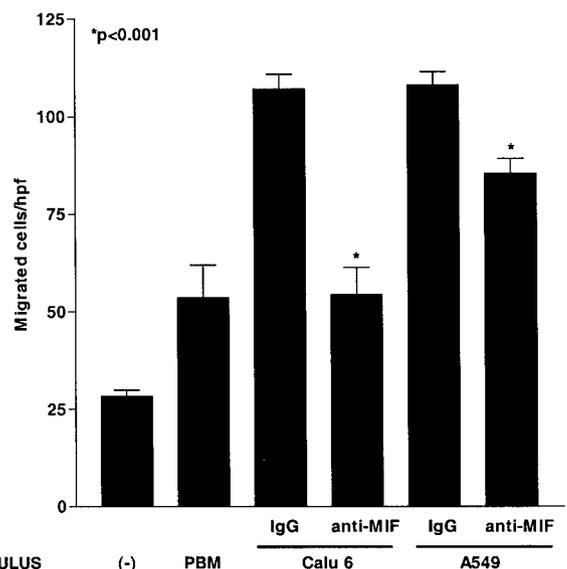

FIGURE 3. Angiogenic activity of monocyte CM after stimulation with NSCLC CM (A549 or Calu 6 cell line) that had been subjected to immunoprecipitation (IP) with anti-MIF Abs or control IgG. PBM alone, 53.5 ± 8.4 cells/hpf; PBM + A549(IgG IP), 108.1 ± 3.4 cells/hpf; PBM + A549 (anti-MIF IP), 85.4 ± 3.2 cells/hpf ($p < 0.001$); PBM + Calu 6 (IgG IP), 107.4 ± 3.8 cells/hpf; PBM + Calu 6 (anti-MIF IP), 54.2 ± 7.1 cells/hpf ($p < 0.001$). Data are representative examples of five experiments.
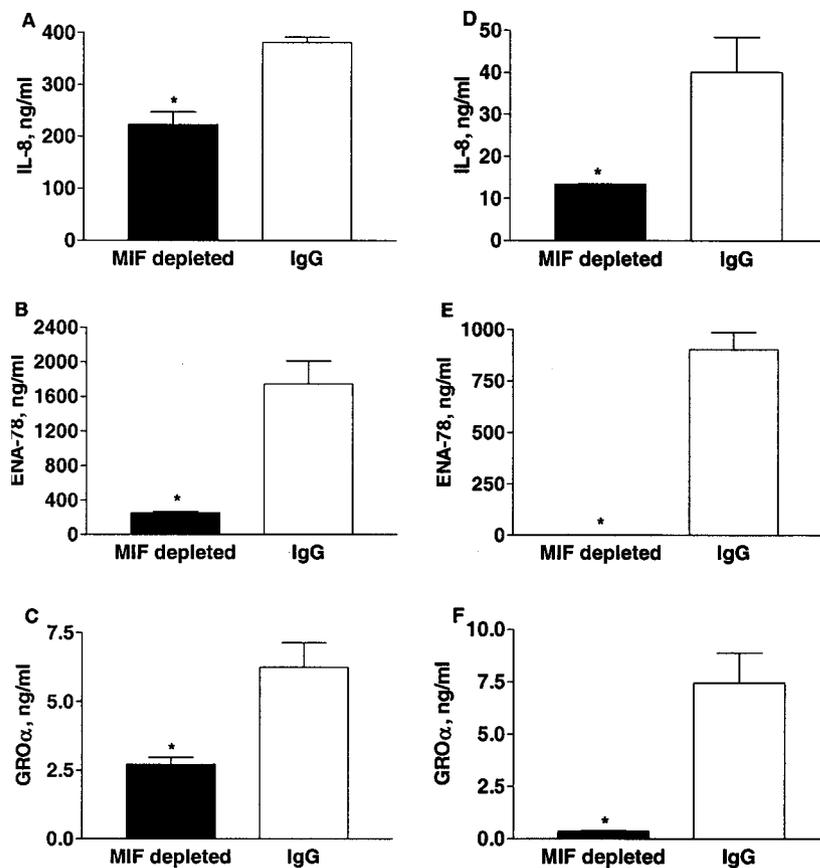
FIGURE 4. CXC chemokine expression from PBM stimulated with MIF-depleted or control A549 CM (*A–C*) or MIF-depleted or control Calu 6 CM (*D–F*). In each condition, MIF-depleted CM induced significantly less expression of PBM-derived angiogenic CXC chemokines than control CM. ∗, $p < 0.05$ for the difference between MIF-depleted CM and control CM for each experiment. Data are representative of two separate experiments.

in significantly decreased CXC chemokine expression compared with control A549 or Calu 6 CM ($p < 0.05$ for each condition; Fig. 4).

Addition of exogenous MIF is sufficient to increase PBM-derived angiogenic CXC chemokine expression

The above data suggests that MIF is necessary for NSCLC cell line-induction of CXC chemokine-dependent angiogenic activity from PBMs. To determine whether MIF was sufficient to induce CXC chemokines, freshly isolated PBM were cultured in six-well plates in the presence of serum-free medium with or without the addition of 10 ng/ml MIF. CM was harvested at 24 h and assayed for IL-8, ENA-78, and GRO-α by specific ELISA. We found that PBM stimulated with MIF expressed significantly higher levels of IL-8 (252.7 ± 16.5 ng/ml vs 54.6 ± 2.0 ng/ml; $p < 0.01$), ENA-78 (264.8 ± 26.9 ng/ml vs 105.2 ± 6.7 ng/ml; $p < 0.01$), and GRO-α (7.9 ± 1.2 ng/ml vs 1.9 ± 0.4 ng/ml; $p < 0.01$) compared with unstimulated PBM (Fig. 5).

Discussion

Tumors require angiogenesis to grow >2–3 mm^3 (1, 9). Many factors are important in tumor angiogenesis, including VEGF (28–30) and CXC chemokines (5, 6, 10, 11). Evidence suggests that a balance between proangiogenic and antiangiogenic factors contributes to net tumor angiogenesis (9, 11, 31). The source(s) of angiogenic factor expression within NSCLC have not been completely elucidated. Malignant cells can and do produce angiogenic factors (22, 32), but recent evidence by Fukumura et al. (14) demonstrates that murine mammary tumors induce VEGF promoter activity in stromal cells. In addition, Anderson and coworkers (32) demonstrated induction of IL-8 (an angiogenic CXC chemokine) in human fibroblasts by malignant cells.

A number of different cell types contribute to angiogenesis, including cancer cells (12, 34), endothelial cells (35–37), fibroblasts (13, 15), eosinophils (38), platelets (39), and monocyte-derived macrophages (40–42). Each cell type displays a certain repertoire of angiogenic factors, depending on the stimulus. Because of the overwhelming redundancy within this system, it is important to determine the cellular source(s) of angiogenic activity in pathologic conditions. In this study, we found that PBM are induced by NSCLC cells to increase expression of angiogenic activity. In addition, we observed some increase in expression of angiogenic factors by NSCLC cells exposed to PBM CM.

Many authors have cited similarities between tumor growth and wound healing (43–45). Macrophages are critically involved in wound repair (46–48). In wounds, local tissue injury results in leakage of plasma and blood from damaged blood vessels resulting in a fibrin clot rich in fibronectin, fibrin breakdown products, and
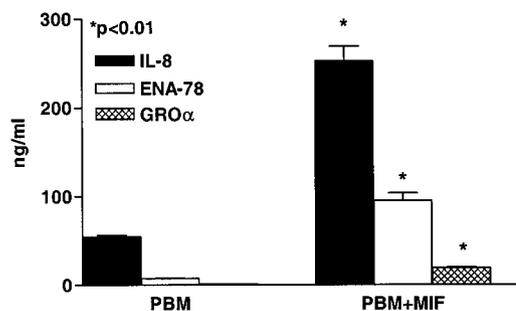

FIGURE 5. CXC chemokine expression from unstimulated PBM (*left*) compared with PBM stimulated with 10 ng/ml MIF (*right*). ∗, $p < 0.01$ for each chemokine comparing unstimulated PBM to MIF stimulated PBM. Data are representative of three separate experiments.

platelets. The fibrin clot becomes a provisional matrix or stroma into which monocytes migrate to ingest wound debris (43). Monocyte-derived macrophages are essential in this role, as evidenced by defective wound repair in macrophage-depleted animals (49). In addition to their role in removing debris, macrophages in inflammatory conditions promote tissue repair by producing cytokines such as TNF-α (50), platelet-derived growth factor (51), and VEGF (52). Analogous events are thought to occur in the process of tumor angiogenesis (43–46).

Polverini and Leibovich (52) demonstrated that tumor-associated macrophages or their CM isolated from experimental fibrosarcoma display angiogenic activity, suggesting the role of macrophage-derived soluble factors in tumor angiogenesis. Similarly, Hildenbrand et al. (53) showed that TGF-β induced macrophages to produce urokinase-plasminogen activator, a known factor in the degradation of tumor matrix and tumor progression. Interestingly, this group also demonstrated that tumor-associated macrophages were induced to produce significantly more urokinase-plasminogen activator than either PBM or elicited tissue macrophages (53), suggesting that the tumor environment can alter macrophage phenotype.

We recently have demonstrated infiltration of monocytes in human NSCLC, which is mediated by tumor expression of CC chemokines (20). Because PBM are a potentially rich source of angiogenic factors, including angiogenic CXC chemokines (41, 55, 56), we hypothesized that NSCLC may augment the expression of macrophage-derived angiogenic factors. Our results show that NSCLC cells promote angiogenesis by inducing host PBM to upregulate CXC chemokine-dependent angiogenic activity. This is important in the context of our previous findings demonstrating the role of the CXC chemokine family in NSCLC-mediated angiogenesis (5, 10, 31). Although it is likely that other factors such as VEGF play an important role in NSCLC angiogenesis, the results of our study do not support a predominant role for VEGF in the monocyte-derived angiogenic activity induced by NSCLC.

Recently, Addison et al. have demonstrated that CXCR2 is the putative receptor for the angiogenic activity mediated by angiogenic CXC chemokines (23). In our study, we found that the increased angiogenic activity in coculture CM could be neutralized by Abs directed against CXCR2. It is interesting to note that the degree of inhibition of angiogenic activity by anti-CXCR2 was comparable to, or only slightly greater than, the inhibition seen with Abs directed at the individual ligands. To account for this observation, one can speculate that Ab binding to an individual CXC chemokine ligand may prevent access to the receptor by other ligands, thereby effectively neutralizing the receptor as well.

We found that MIF, expressed constitutively by the A549 and Calu 6 cell lines, is responsible in large part for the induction of PBM-derived angiogenic activity. MIF was initially discovered as an activity released by cells in delayed-type hypersensitivity reactions. Recently, a variety of immunomodulatory actions have been attributed to MIF (56). Interestingly, several groups of investigators have found that MIF can promote angiogenic activity in mouse models of tumor growth (25, 26). However, data demonstrating direct angiogenic activity for MIF is lacking. These reports prompted us to investigate whether NSCLC cells expressed MIF and whether this might be a mechanism by which NSCLC cells induce PBM-derived angiogenic activity. We found constitutive expression of MIF protein in the CM of A549 and Calu 6 cells. Furthermore, we demonstrated that MIF was responsible in part for the induction of PBM-derived CXC chemokine-dependent angiogenic activity by NSCLC cells.

We observed that the degree of PBM stimulation by NSCLC cell lines was greater when CM was used as the stimulus compared with coculture conditions. It is interesting to speculate on the existence of a simultaneous inhibitory signal generated by NSCLC cells and present in soluble form. This inhibitory signal must be postulated to have a short biologic half-life in vitro, given that its effect is only seen in coculture conditions. When CM is generated separately and used to stimulate PBMs, a mediator of short half-life would have less of an effect in the absence of continued secretion.

Combined with our previous findings of CC chemokine-mediated monocyte infiltration of NSCLC tumors (20), our current data suggest that tumors orchestrate a complex cytokine network in which macrophages recruited to the tumor site are influenced to promote a proangiogenic milieu. Our findings may account for the antitumor activity of neutralizing Abs to MIF in animal models of cancer as reported by other investigators (25–27).

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