Interferon-γ-inducible Protein 10 (IP-10) Is an Angiostatic Factor That Inhibits Human Non-small Cell Lung Cancer (NSCLC) Tumorigenesis and Spontaneous Metastases

By Douglas A. Arenberg,* Steven L. Kunkel,§ Peter J. Polverini,‡ Susan B. Morris,* Marie D. Burdick,* Mary C. Glass,* Dennis T. Taub,‖ Mark D. Iannettoni,¶ Richard I. Whyte,¶ and Robert M. Strieter*

*From the *Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, University of Michigan Medical School, Ann Arbor, Michigan 48109-0360; ‡Section of Oral Pathology, University of Michigan Dental School, Ann Arbor, Michigan 48109-1078; §Department of Pathology, University of Michigan, Ann Arbor, Michigan 48109; ‖Laboratory of Molecular Immunoregulation, National Cancer Institute, Frederick, Maryland 21702; ¶Department of Surgery, Section of Thoracic Surgery, University of Michigan, Ann Arbor, Michigan 48109*

Summary

The success of solid tumor growth and metastasis is dependent upon angiogenesis. Neovascularization within the tumor is regulated, in part, by a dual and opposing system of angiogenic and angiostatic factors. We now report that IP-10, a recently described angiostatic factor, is a potent angiostatic factor that regulates non-small cell lung cancer (NSCLC)-derived angiogenesis, tumor growth, and spontaneous metastasis. We initially found significantly elevated levels of IP-10 in freshly isolated human NSCLC samples of squamous cell carcinoma (SCCA). In contrast, levels of IP-10 were equivalent in either normal lung tissue or adenocarcinoma specimens. The neoplastic cells in specimens of SCCA were the predominant cells that appeared to express IP-10 by immunolocalization. Neutralization of IP-10 in SCCA tumor specimens resulted in enhanced tumor-derived angiogenic activity. Using a model of human NSCLC tumorigenesis in SCID mice, we found that NSCLC tumor growth was inversely correlated with levels of plasma or tumor-associated IP-10. IP-10 in vitro functioned as neither an autocrine growth factor nor as an inhibitor of proliferation of the NSCLC cell lines. Reconstitution of intratumor IP-10 for a period of 8 wk resulted in a significant inhibition of tumor growth, tumor-associated angiogenic activity and neovascularization, and spontaneous lung metastases; whereas, neutralization of IP-10 for 10 wk augmented tumor growth. These findings support the notion that tumor-derived IP-10 is an important endogenous angiostatic factor in NSCLC.

Lung cancer is responsible for ~150,000 deaths per year in the US (1–3). Treatment protocols for non-small cell lung cancer (NSCLC)[1] currently yield an overall 5 yr survival rate of 15% (1, 4). To develop more effective therapy, further insights into the biology of NSCLC are required. NSCLC, similar to other solid tumors, is dependent upon neovascularization to continually supply the tumor with nutrients and oxygen (5, 6). Investigations of tumor angiogenesis have primarily focused on the role of angiogenic factors, such as vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), transforming growth factor β (TGFβ), and basic fibroblast growth factor (bFGF) (7–10). However, recent studies have demonstrated an increasing role for endogenous angiostatic factors in the regulation of net tumor-associated neovascularization. For example, the loss of endogenous angiostatic molecules may contribute to the augmentation of tumor-associated angiogenic activity that enhances tumorigenesis and potential spontaneous metastasis (11–14). This suggests that overall tumor-derived neovascularization is regulated by an imbalance in the expression of angiogenic and angiostatic factors present within the tumor.

Historically, interferons have been shown to inhibit tumor growth and wound repair (15, 16). One of the mechanisms for this effect appears to be related to their angiostatic properties (17–19). Interferon-γ-inducible protein 10 (IP-10) is a CXC chemokine produced by a wide variety

[1] *Abbreviations used in this paper:* BCE, bovine adrenal gland capillary endothelial cells; bFGF, basic fibroblast growth factor; HPF, high power fields; IP-10, interferon-γ-inducible protein 10; MIG, monokine induced by interferon-gamma; NSCLC, non-small cell lung cancer; SCCA, squamous cell carcinoma; VEGF, vascular endothelial growth factor.

of cells in response to interferons (20–23). Our laboratory and others have recently identified that IP-10 is a potent angiostatic factor (24–27), suggesting that IP-10 may be a distal mediator of the angiostatic effects of interferons.

We postulated that IP-10 is an important endogenous inhibitor of angiogenesis in human NSCLC. To test this hypothesis, we initially isolated fresh specimens of human NSCLC, and found that squamous cell carcinoma (SCCA), as compared to either normal lung tissue or adenocarcinoma specimens, was associated with increased levels of IP-10. Neutralization of IP-10 from these samples demonstrated that IP-10 behaved as a potent endogenous inhibitor of SCCA-derived angiogenic activity. To extend these observations to an in vivo model system, a human NSCLC/SCID mouse chimera was employed by injecting human NSCLC cell lines, A549 (adenocarcinoma) and Calu 1 (squamous cell carcinoma), into the flanks of SCID mice. We found that the production of tumor-derived IP-10 was inversely correlated with the rate of growth of the two human NSCLC cells lines. IP-10 functioned as neither an autocrine growth factor nor as an inhibitor of in vitro proliferation of the NSCLC cell lines. Moreover, an in vivo strategy to reconstitute intratumor IP-10, by injecting tumors with recombinant human IP-10, as compared to an equimolar concentration of an irrelevant human protein, resulted in a decrease in tumorigenesis, tumor-derived angiogenic activity and neovascularization, and spontaneous lung metastases. This effect was unrelated to any alteration of tumor infiltrating leukocytes. Moreover, depletion of IP-10 for 10 wk resulted in augmented tumor growth. These findings suggest that IP-10 is an important endogenous angiostatic factor in human NSCLC, specifically SCCA, and illustrates a potential strategy to correct the imbalance of angiogenic activity in human NSCLC.

Materials and Methods

Reagents. Polyclonal rabbit anti-human IP-10 sera was produced by immunization of rabbits with recombinant human IP-10 (Peprotech, Rocky Hill, NJ) in multiple intradermal sites with complete Freund's adjuvant. The specificity of the IP-10 antiserum was confirmed by ELISA and Western blot analysis, and was not cross-reactive with a panel of 12 other recombinant human cytokines or the murine chemokines KC and MIP-2. In addition, this antisera recognized both murine and human forms of IP-10 using Western blot analysis, and was endotoxin-free. Rabbit anti-Factor VIII-related antigen antibodies used for FACS® analysis of tumor-associated vasculature were purchased from Biomeda (Foster City, CA). Rabbit anti-murine macrophage and neutrophil antibodies were purchased from Accurate Antibodies (Westbury, NY). A549 (adenocarcinoma) cell culture media consisted of RPMI-1640 (Whitaker Biomedical Products, Whitaker, CA) supplemented with 1 mM glutamine, 25 mM Hepes buffer, 100 U/ml penicillin, 100 ng/ml streptomycin, and 10% fetal calf serum. Calu 1 (SCCA) cell culture media consisted of Eagle's minimal essential medium, supplemented with 1 mM glutamine, 25 mM Hepes buffer, 100 U/ml penicillin, 100 ng/ml streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, and 10% fetal calf serum. The "anti-protease" buffer for tissue homogenization consisted of 1× PBS with 2 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of antipan, aprotinin, leupeptin, and pepstatin A.

Normal Human Lung and NSCLC Tumor Tissue. Tissue specimens were obtained from consented individuals undergoing thoracotomy (n = 90) for suspected NSCLC in accordance with the University of Michigan I.R.B. approval. Samples of tumor, and normal lung distal to tumor, were homogenized and sonicated in "anti-protease" buffer upon recovery from the operating room. Specimens were centrifuged at 900 g for 15 min, filtered through 0.45 μm Sterile Acrodiscs (Gelman Sciences, Ann Arbor, MI), and frozen at −70°C until thawed for assay by a specific IP-10 ELISA. A portion of this specimen was used for the in vitro endothelial cell chemotaxis assay; and another portion was lyophilized (SpeedVac; Savant, Farmingdale, NY), normalized to an equivalent amount of total protein, and used in the corneal micropocket model of neovacularization for analysis of angiogenic activity. Additionally, a portion of tumor was also fixed in 4% paraformaldehyde and imbedded in paraffin for histologic and immunohistochemical analysis. Histologic determination of NSCLC cell type was provided by University of Michigan Pathologists. Only cases of NSCLC were included in our analysis, (squamous cell carcinoma, n = 40; adenocarcinoma, n = 50). Normal lung tissue was examined histologically and used as a control only if the histology appeared normal without evidence for inflammation or malignant cell invasion (n = 49).

IP-10 ELISA. Antigenic IP-10 was quantitated using a modification of a double ligand method as previously described (28, 29). Briefly, flat-bottomed 96-well microtiter plates (Nunc Immuno-Plate I 96-F; Fisher Scientific, Pittsburgh, PA) were coated with 50 μl/well of the polyclonal anti-IP-10 (1 ng/μl in 0.6 M NaCl, 0.26 M H_3B0_4, and 0.08 N NaOH, pH 9.6) for 24 h at 4°C and then washed with phosphate-buffered saline (PBS), pH 7.5, 0.05% Tween-20 (wash buffer). Microtiter plate nonspecific binding sites were blocked with 2% BSA in PBS and incubated for 60 min at 37°C. Plates were rinsed three times with wash buffer. 50 μl of either tumor homogenate or plasma were added (neat and 1:10 dilution), followed by incubation for 1 h at 37°C. Plates were washed three times, 50 μl/well of biotinylated polyclonal rabbit anti-IP-10 (3.5 ng/μl in PBS, pH 7.5, 0.05% Tween-20, and 2% FCS) added, and plates incubated for 45 min at 37°C. Plates were washed three times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) added, and the plates incubated for 30 min at 37°C. Plates were washed three times and chromogen substrate (Bio-Rad Laboratories) added. The plates were incubated at room temperature to the desired extinction, and the reaction terminated with 50 μl/well of 3 M H_2SO_4 solution. Plates were read at 490 nm in an automated microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Standards were dilutions of IP-10 from 100 ng/ml to 1 pg/ml (50 μl/well). This protocol consistently detected IP-10 concentrations greater than 50 pg/ml in a linear fashion. Tissue samples were run in parallel for total protein (TP) content (Pierce Chem. Co., Rockford, IL), and results expressed as ng of IP-10 per mg TP.

Immunolocalization of IP-10 Protein. IP-10 protein was immunolocalized in paraffin-embedded human NSCLC tissue using a modification of our previously described technique (30, 31). Briefly, paraffin-embedded tissue sections were de-waxed with xylene, and rehydrated through graded concentrations of ethanol. Nonspecific protein binding sites were blocked using normal goat serum (BioGenex, San Ramon, CA). Tissue sections were then washed and incubated with 1:1,000 diluted rabbit anti-IP-10 serum, or an equivalent dilution of control antisera. The tissue sec-

tions were washed and then incubated for 60 min with biotinylated goat anti-rabbit antibodies (BioGenex). Sections were then washed twice in TRIS-buffered saline and incubated with alkaline phosphatase conjugated to streptavidin (BioGenex). Fast Red (BioGenex) reagent was used for chromogenic localization of IP-10 antigen. After optimal color development, tissue sections were immersed in sterile water, counter-stained with Mayer's hematoxylin, and cover slipped using an aqueous mounting solution.

Endothelial Cell Chemotaxis Assay. Endothelial cell chemotaxis was performed in 48-well, blind well chemotaxis chambers (Costar Corp., Cambridge, MA) as previously described (32). Briefly, bovine adrenal gland capillary endothelial cells (BCE) were suspended at a concentration of 10^6 cells/ml in DME with 0.1% BSA and placed into each of the bottom wells (25 ml). Nucleopore chemotaxis membranes (5 μm pore size) were first prepared by soaking in 3% acetic acid for 12 h, followed by coating for 2 h in gelatin (0.1 mg/ml). The membranes were rinsed in sterile water, dried under a laminar flow hood, and stored at room temperature for up to 1 mo. The membranes were placed over the wells, chambers sealed, inverted, and incubated for 2 h to allow cells to adhere to the membrane. Chambers were then reinverted, and 50 ml of human NSCLC tumor homogenates normalized to TP and pre-incubated in the presence of either 1:1,000 dilution of control or neutralizing rabbit anti–human IP-10 antibodies were placed in the top wells and reincubated for an additional 2 h. Membranes were then fixed in methanol, stained with a Diff-Quick staining kit (American Scientific Products), and the cells which had migrated through the membrane were counted in ten high power fields (HPF; 400×). Results were expressed as the number of endothelial cells that had migrated per HPF. Each sample was assessed in triplicate. Experiments were repeated at least three times.

Corneal Micropocket Assay of Angiogenesis. Angiogenic activity of NSCLC tumor homogenates was assayed in vivo in the avascular cornea of hooded Long-Evans female rat eyes, as previously described (33, 34). Briefly, equal volumes of lyophilized tumor specimens normalized to total protein, were combined with sterile Hydron (Interferon Sciences Inc., New Brunswick, NJ) casting solution. 5-μl aliquots were pipetted onto the flat surface of an inverted sterile polypropylene specimen container, and polymerized overnight in a laminar flow hood under UV light. Before implantation, pellets were rehydrated with normal saline. Animals were anesthetized with ketamine (150 mg/kg) and atropine (250 μg/kg) i.p. Rat corneas were anesthetized with 0.5% proparacaine hydrochloride ophthalmic solution followed by implantation of the Hydron pellet into an intracorneal pocket (1 to 2 mm from the limbus). 6 d after implantation, animals received 1,000 U of heparin and ketamine (150 mg/Kg) i.p., followed by a 10-ml perfusion of colloidal carbon via the left ventricle. Corneas were harvested and photographed. No inflammatory response was observed in any of the corneas treated with the above specimens. Positive neovascularization responses were recorded only if sustained directional ingrowth of capillary sprouts and hairpin loops towards the implant were observed. Negative responses were recorded when either no growth was observed or when only an occasional sprout or hairpin loop displaying no evidence of sustained growth was detected. All animals were handled in accordance with the University of Michigan unit for laboratory animal medicine (ULAM).

Human NSCLC Cell Lines. The A549 (adenocarcinoma) and Calu 1 (squamous cell carcinoma) cell lines (American Type Culture Collection, Rockville, MD) were maintained in sterile 150 cm^2 tissue culture flasks. Cells were cultured and passaged at 37°C in room air/5% CO_2. For proliferation assays, 2×10^3 cells were plated in each well of a 96-well plate and allowed to grow for 24 h, serum starved for 24 h, then treated with varying concentrations of IP-10 in media with 1% serum. After 24 and 48 h, cells were then quantitated using a non-radioactive cell proliferation assay according to the manufacturers instructions (CellTiter 96 AQ; Promega Corp., Madison, WI) and using a standard (0, 10^3, 2×10^3, 5×10^3, 7.5×10^3, 10^4, 2.0×10^4, 2.5×10^4, 5×10^4, 7.5×10^4, 1×10^5, 2×10^5) of NSCLC cells on the day of assay. For inoculation into mice, the cells were trypsinized, harvested, washed, and resuspended in serum-free media at a concentration of 1×10^7 cells/ml.

Human NSCLC-SCID Mouse Chimeras. 4–6-wk-old female CB17-SCID mice (Taconic Farms, Germantown, NY) with serum Ig <1 μg/ml were injected subcutaneously with human NSCLC cells (1×10^6 cells in 100 μl) into each flank. The animals were maintained under sterile conditions in laminar flow rooms. Tumor-bearing mice were killed at weekly intervals (n = 6 per wk). At time of death, anticoagulated (heparin 50 U/500 μl of blood) blood was collected and centrifuged. The isolated plasma was stored at −70°C for later analysis. In addition, at time of death tumors were dissected from the mice, weighed, and measured with a Thorpe caliper (Biomedical Research Instruments, Rockville, MD). The lungs were inflated with 4% paraformaldehyde, and fixed in paraffin. In the IP-10 treatment studies, mice received intra-tumor injections of either human recombinant IP-10 (1 μg in 20 μl of sterile saline every other day) or an equimolar concentration of an irrelevant protein (human serum albumin; HSA) beginning at the time of tumor inoculation. In the IP-10 depletion studies, SCID mice received intraperitoneal injections of 500 μl of either neutralizing rabbit anti-human IP-10 or control (pre-immune) serum every 48 h for 10 wk, starting at the time of cell inoculation. All animals were handled in accordance with ULAM.

Processing of SCID/NSCLC Tumor Specimens. Human NSCLC tumor specimens from SCID mice were processed as follows. One portion was homogenized and sonicated in "anti-protease" buffer, centrifuged at 900 *g* for 15 min, filtered through 0.45 μm Sterile Acrodiscs (Gelman Sciences), and frozen at −70°C until thawed for assay by a specific IP-10 ELISA. Another portion was fixed in 4% paraformaldehyde for histologic analysis and immunohistochemistry. The final portion of tumor was minced into <1 mm^3 sections and incubated for 1 h in protease digestion media (Dispase, Collaborative Biomedical Products, Two Oak Park, MA). Cells were then pelleted at 600 *g* for 10 min, red blood cells lysed, washed in PBS, and resuspended in complete media with 5% FCS. Cells were counted, and transferred to fluorescent antibody buffer (1% FA buffer; Difco Detroit, MI, 1% FCS, and 0.1% azide, 5×10^6 cells/ml) and maintained at 4°C for the remainder of the staining procedure. 100 μl of tumor cells were labeled with rabbit anti-Factor VIII-related antigen antibodies to recognize tumor-associated endothelial cells. Pre-immune rabbit-IgG was used as a control. FITC-conjugated goat anti–rabbit IgG was used as a secondary antibody. FACS® analysis was then employed to detect Factor VIII-related antigen expressing cells. Tumor vasculature was expressed as the percentage of cells from the tumor that were positive for Factor VIII-related antigen.

Quantitation of Infiltrating Leukocytes. Leukocytes were localized and quantitated in human NSCLC tumor/SCID mouse specimens treated with either IP-10 or HSA using a modification of our previously described method (30, 31). Briefly, tissue sections were de-waxed with xylene, and rehydrated through graded concentrations of ethanol. Nonspecific protein binding sites were blocked using normal goat serum (BioGenex). Sections were

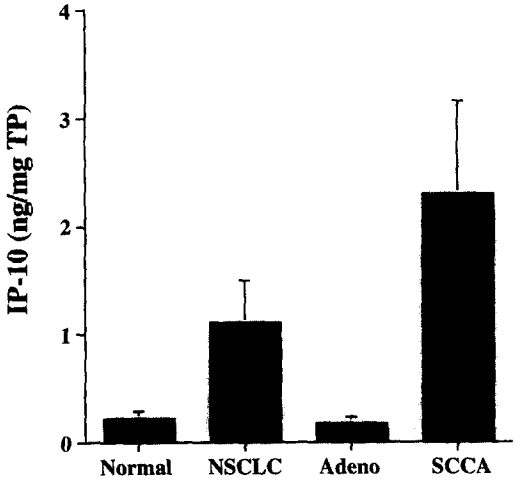

Figure 1. NSCLC-derived IP-10 protein. IP-10 measured from either normal lung tissue, NSCLC (combined specimens of adenocarcinoma [*Adeno*] and squamous cell carcinoma [*SCCA*]), adeno (n = 50) or SCCA (n = 40) cell-types of NSCLC. IP-10 is expressed as ng/mg of total protein (*TP*).

then washed and incubated with either rabbit anti-murine macrophage (1:1,000), rabbit anti-murine neutrophil antisera (1:1,000), or an equivalent dilution of control antisera. The tissue sections were washed and then incubated for 60 min with biotinylated goat anti-rabbit antibodies (BioGenex). Sections were then washed twice in TRIS-buffered saline and incubated with alkaline phosphatase conjugated to streptavidin (BioGenex). Fast Red (BioGenex) reagent was used for chromogenic localization of murine leukocytes. After optimal color development, tissue sections were immersed in sterile water, counter-stained with Mayer's hematoxylin, and cover slipped using an aqueous mounting solution. Tumor sections were then examined under 200× magnification and cells counted. A total of five fields per tumor section, six sections per tumor, and six tumors per treatment group were counted. Results were expressed as the number of leukocytes per high power field (200×).

Quantitation of Lung Metastases by Light Microscopy. Six H&E stained lung sections from each lung of SCID mice treated with either intratumor IP-10 or HSA for 8 wk (n = 6 animals per group) were examined under low magnification (40×) for evidence of spontaneous metastases and counted. In addition, using an Olympus BH-2 microscope coupled to a Sony 3CCD camera

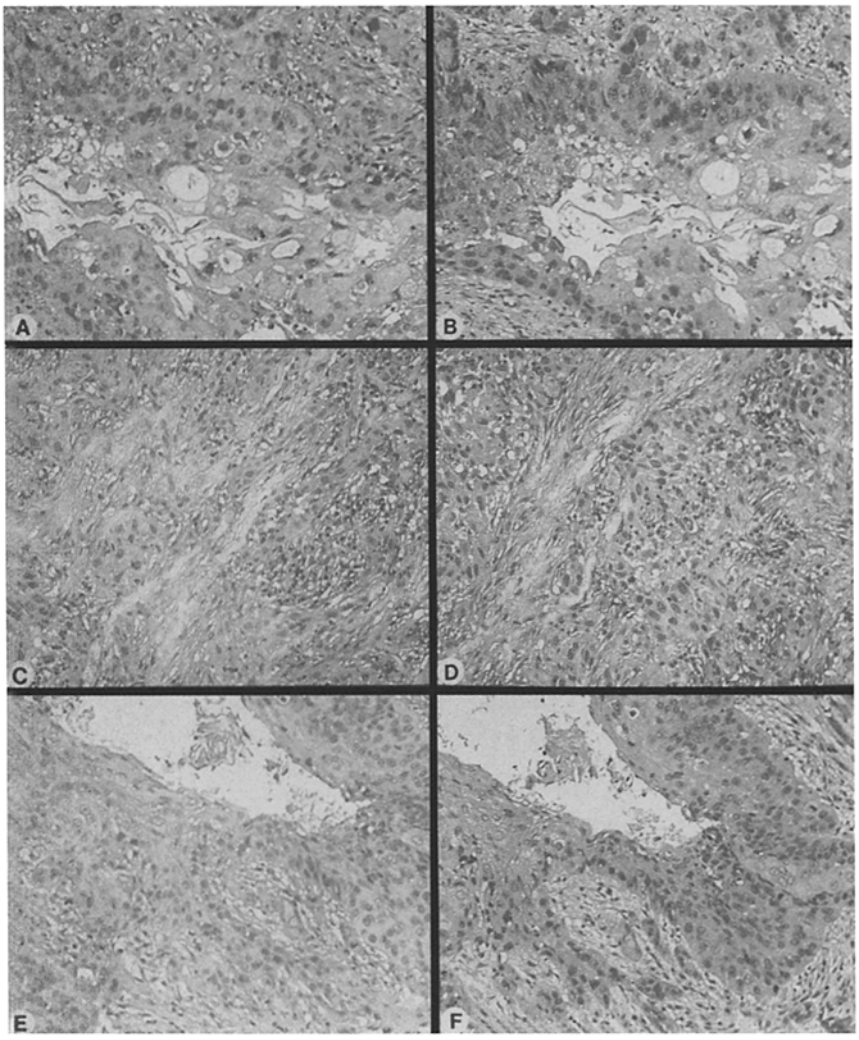

Figure 2. Representative photomicrographs of the immunolocalization of IP-10 in squamous cell carcinoma (SCCA). *A*, *C*, and *E* represent control staining of SCCA with pre-immune antibodies (200×). *B*, *D*, and *F* represent staining with anti-IP-10 antibodies (200×).

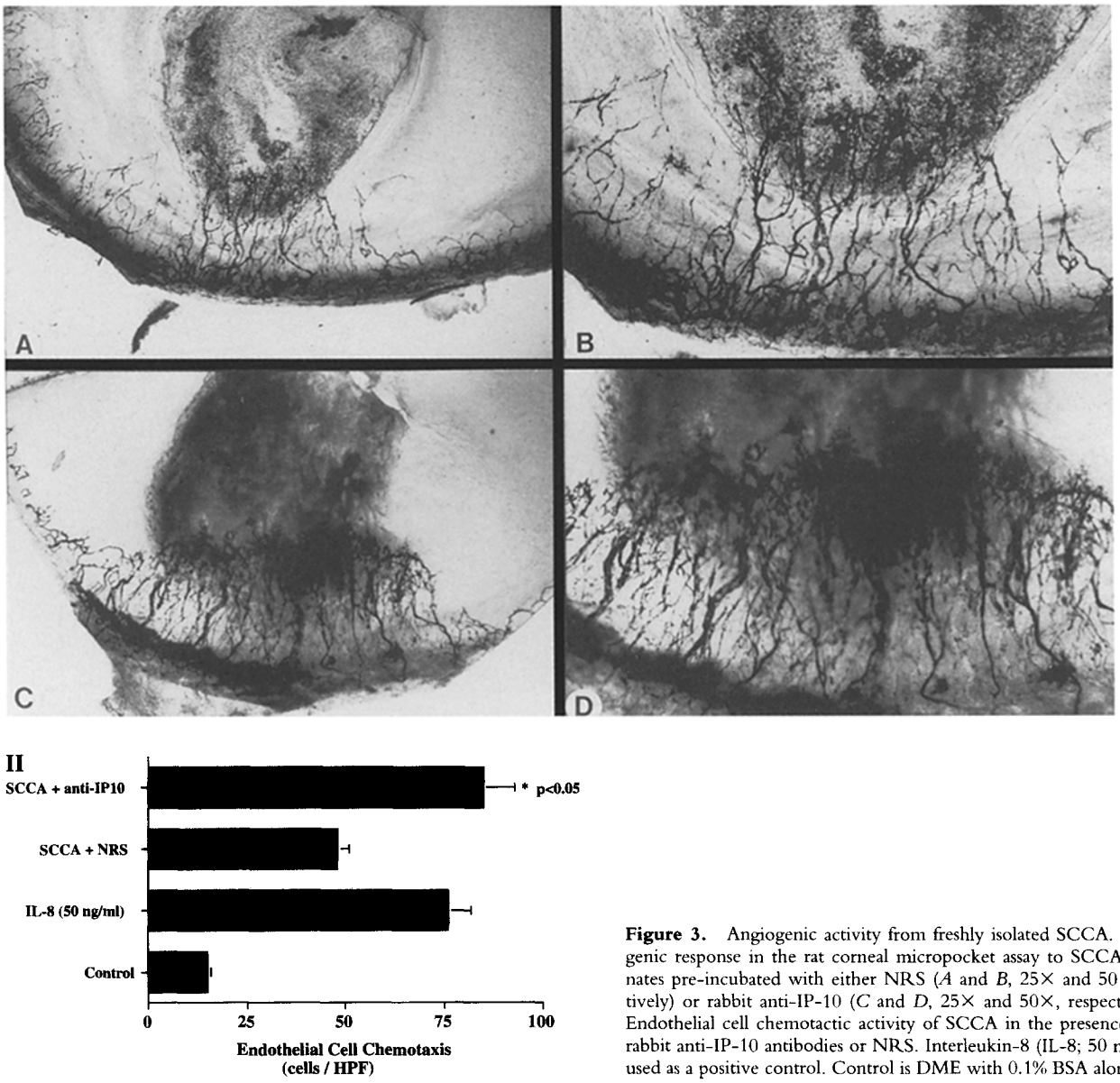

Figure 3. Angiogenic activity from freshly isolated SCCA. (*I*) Angiogenic response in the rat corneal micropocket assay to SCCA homogenates pre-incubated with either NRS (*A* and *B*, 25× and 50×, respectively) or rabbit anti-IP-10 (*C* and *D*, 25× and 50×, respectively). (*II*) Endothelial cell chemotactic activity of SCCA in the presence of either rabbit anti-IP-10 antibodies or NRS. Interleukin-8 (IL-8; 50 ng/ml) was used as a positive control. Control is DME with 0.1% BSA alone.

and a Macintosh IIfx computer the total area of metastatic tumor burden per lung section was quantitated using NIH Image 1.55 software. Data were expressed as either the number of metastases per lung section or the area of metastatic tumor per section (square pixels at 40× magnification).

Statistical Analysis. The animal studies involved a minimum of 12 human NSCLC tumors or 6 SCID mice at each time point or for each manipulation. Groups of data were evaluated by analysis of variance to indicate groups with significant differences. Data that appeared statistically significant were compared by Student's *t* test for comparing the means of multiple groups, and were considered significant if *P* values were less than 0.05. The Mann-Whitney U test was used to compare groups of observations which were not normally distributed. Results are presented as means ± SEM. Data was analyzed by Macintosh IIfx computer using Statview 4.5 statistical software package (Abacus Concepts, Inc.).

Results

IP-10 Is Significantly Elevated in Human NSCLC. To determine whether IP-10 protein was present in human NSCLC, freshly isolated specimens of bronchogenic tumors were assessed by specific IP-10 ELISA (Fig. 1). The levels of IP-10 from tumor specimens were significantly higher than in normal lung tissue (1.11 ± 0.39 vs. 0.23 ± 0.07 ng/mg TP, $P < 0.05$). To ascertain whether the presence of IP-10 protein varied by histologic cell-type, results were further subdivided by cell-type (SCCA vs. adenocarcinoma). The increase in IP-10 from NSCLC tissue was entirely attributable to the higher levels of IP-10 present in SCCA, as compared to adenocarcinoma (2.25 ± 0.83 vs. 0.19 ± 0.06 ng/mg TP, $P < 0.05$, Fig. 1). Immunolocalization of IP-10 protein within SCCA specimens revealed

that the predominant cellular source of IP-10 was the neoplastic cells (Fig. 2).

IP-10 Is an Endogenous Angiostatic Factor in NSCLC (SCCA). While the above experiments demonstrated that IP-10 protein was significantly elevated in specimens of freshly isolated SCCA, we postulated that IP-10 may be acting in vivo to regulate tumor-derived angiogenesis. To test this hypothesis, we preincubated specimens of human SCCA normalized to TP in the presence of either control or neutralizing antibodies to IP-10 and assessed their angiogenic activity using either in vivo corneal neovascularization or in vitro endothelial cell chemotaxis (Fig. 3, *I* and *II*, respectively). SCCA samples preincubated with neutralizing IP-10 antibodies, as compared to control antibodies demonstrated a significant increase in their endothelial cell chemotactic activity (85.0 ± 8.0 vs. 48.0 ± 3.0 cells/hpf, $P < 0.05$) (Fig. 3 *II*). These findings were further confirmed using the in vivo rat corneal micropocket assay of neovascularization, as SCCA specimens preincubated in the presence of neutralizing antibodies to IP-10 (Fig. 3 *I*, *C* and *D*, at a magnification of 25× and 50×, respectively), as compared to control antibodies (Fig. 3 *I*, *A* and *B*, at a magnification of 25× and 50×, respectively), demonstrated an augmented neovascular response in the cornea.

The Production of IP-10 Inversely Correlates with Tumorigenesis of Human NSCLC in SCID Mice. The above findings suggested that IP-10 represents an important endogenous angiostatic factor in freshly isolated NSCLC (SCCA) of the lung. However, to determine if this angiostatic activity was physiologically relevant during the course of in vivo tumor growth, a human NSCLC/SCID mouse model of human NSCLC tumorigenesis was employed. SCID mice were inoculated with either A549 (adenocarcinoma) or Calu 1 (SCCA) cells (1 ± 10^6 cells/flank) and killed weekly beginning with the second and third week of tumor growth, respectively. The experiments using A549 cells were terminated at 8 wk due to morbidity noted in the animals secondary to tumor burden. As shown in Fig. 4 *A*, there was a progressive increase in tumor size in A549-bearing animals beginning at week 2 through week 8. In contrast, animals bearing Calu 1 tumors demonstrated little growth until week 8 (Fig. 4 *A*). The production of IP-10 from A549 and Calu 1 tumors was inversely correlated with tumor growth ($r = -0.648$ and -0.688 for A549 and Calu 1 tumors, respectively, $P < 0.05$)(Fig. 4 *B*). In addition, IP-10 levels were significantly higher in the Calu 1 (SCCA) tumors, as compared to A549 tumors. Plasma IP-10 levels from tumor-bearing SCID mice paralleled the findings from the primary tumors (data not shown). Furthermore, the appearance of spontaneous lung metastases in SCID mice bearing A549 tumors occurred after IP-10 levels from either the primary tumor or plasma reached a nadir. To determine whether IP-10 in vitro was an autocrine growth factor for these cell lines, A549 and Calu 1 cells were cultured in the presence or absence of recombinant IP-10 for 24 and 48 h (Table 1, and data not shown, respectively). The presence of exogenous IP-10 did not alter proliferation, as compared to appropriate controls ($P > 0.2$). These findings suggested that IP-10 neither functions as an autocrine growth factor nor an inhibitor of cellular proliferation of human NSCLC cell lines.

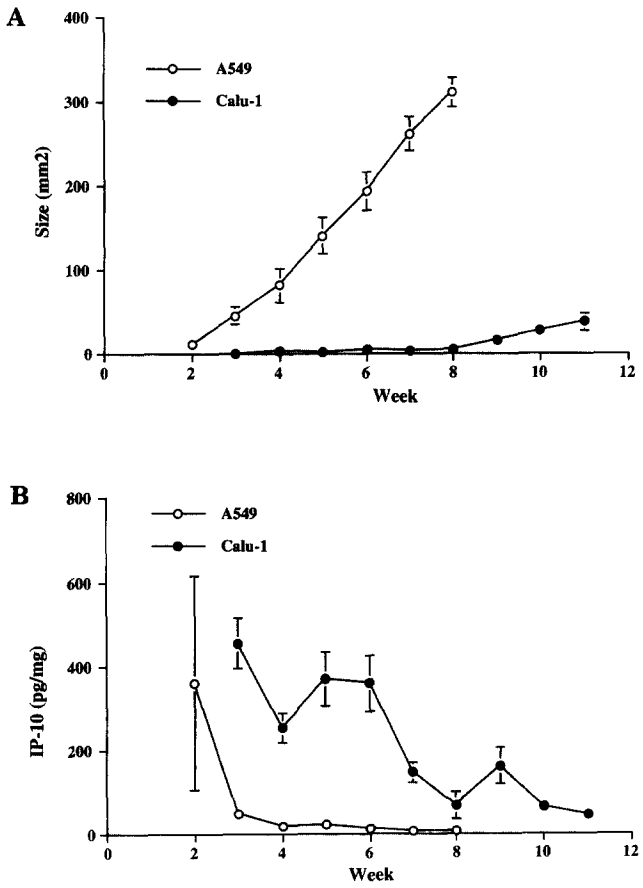

Figure 4. Tumorigenesis of human non-small cell lung cancer, adenocarcinoma (*A549*) and squamous cell carcinoma (*Calu-1*), in SCID mice as it correlates to the production of tumor-derived IP-10. (*A*) Tumor growth as measured by size (mm^2). (*B*) the kinetics of tumor-derived IP-10 as expressed as pg/mg of total protein (*TP*).

The Presence of Intratumor IP-10 Attenuates or Absence of IP-10 Augments Tumor Growth and Spontaneous Lung Metastases. Since IP-10 was found to be a potent endogenous angiostatic molecule in SCCA, the reduced expression of IP-10 in A549 (adenocarcinoma) tumors, as compared to Calu 1 (SCCA) tumors, may contribute to their more aggressive behavior. We hypothesized that restoration of tumor associated IP-10 in A549 tumors could lead to inhibition of tumorigenesis via an IP-10-dependent decrease in tumor-associated angiogenic activity and neovascularization. SCID mice bearing A549 tumors were injected (intratumor) with either recombinant human IP-10 (1 μg in 20 μl of normal saline) or 20 μl of an equimolar concentration of an irrelevant human protein, human serum albumin (HSA), every 48 h for a period of 8 wk beginning at the time of tumor cell inoculation. The intratumor administration of IP-10 resulted in a 40% and 42% reduction in tumor size and mass, respectively, as compared to tumors treated with intratumor HSA (0.79 ± 0.14 g vs. 1.37 ± 0.23 g and

Table 1. *Proliferation of NSCLC Cell Lines (A549 and Calu 1) in the Presence or Absence of IP-10 for 24 h*

	Stimulation			
IP-10	0 ng/ml	1 ng/ml	10 ng/ml	100 ng/ml
		cells (10^4)		
A549	20 ± 4.0	36 ± 6.0	33 ± 4.0	28 ± 4.0
Calu 1	38 ± 4.0	39 ± 6.0	39 ± 3.0	41 ± 3.0

136 ± 17 mm^2 vs. 217 ± 27 mm^2 at 8 wk, respectively, $P \leq 0.03$, Fig. 5, *A–C*). To exclude that IP-10 inhibited tumor growth by recruiting tumoricidal leukocytes, quantitation of tumor-infiltrating leukocytes was performed. A549 tumors from SCID mice treated for 8 wk with IP-10, as compared to HSA, revealed no evidence for alterations in intratumor leukocyte populations (Fig. 6). We sought to determine whether IP-10 treatment of the primary tumor also reduced spontaneous lung metastases. The number of metastases was significantly reduced in mice treated with IP-10, as compared with HSA (3.5 ± 0.6 vs. 8.7 ± 0.9 metastases per lung section, respectively, $P < 0.001$, Fig. 7 *A*). In addition, the size (area) of the lung metastases per section was also dramatically reduced in the IP-10, as compared to the HSA treated mice (37 ± 13 × 10^3 vs. 142 ± 29 × 10^3 square pixels, respectively, $P < 0.01$, Fig. 7 *B*). To further demonstrate the importance of endogenous IP-10 in the regulation of human NSCLC (SCCA) tumor growth, we passively immunized SCID mice bearing Calu 1 tumors with either neutralizing rabbit anti-human IP-10 or control antibodies for 10 wk. Calu 1 tumors from animals that were passively immunized with neutralizing antibodies to IP-10 for 10 wk demonstrated a 1.8–2.9-fold increase in tumor size, as compared to tumors from animals that had received control antibodies (data not shown). However, there was no evidence of lung metastases in either group.

Reconstitution of Intratumor IP-10 Attenuates Tumor-associated Angiogenic Activity and Vasculature. To further determine the mechanism of growth inhibition by intratumor administration of IP-10, we directly evaluated angiogenic activity from A549 tumors of animals that had been treated in vivo with either IP-10 or HSA for 8 wk. The previously well-characterized rat corneal micropocket assay of neovascular-

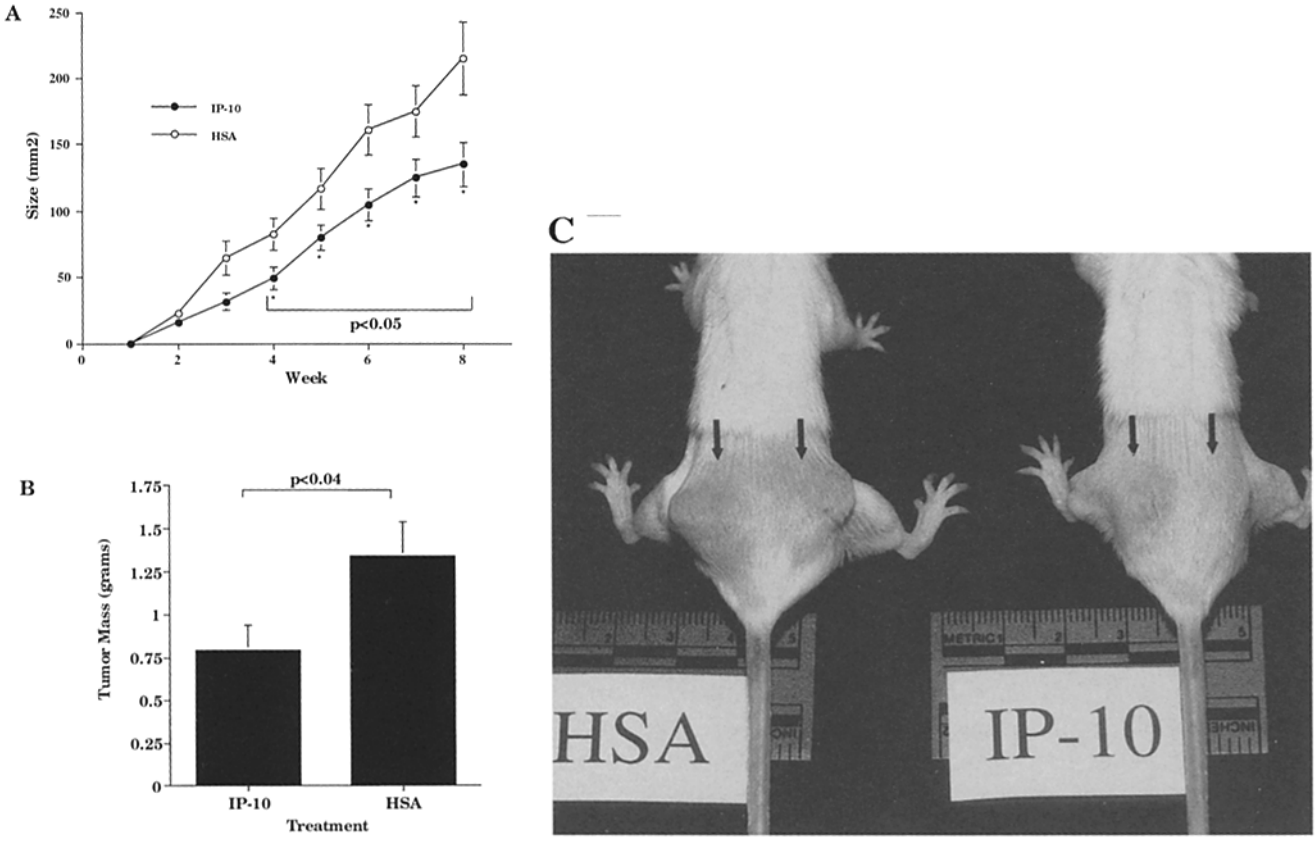

Figure 5. Intratumor injection of IP-10 inhibits growth of human adenocarinoma (A549) tumors in SCID mice. (*A*) IP-10-induced inhibition of tumor growth, as compared to tumors treated with human serum albumin (*HSA*). (*B*) The difference in tumor mass (grams) at 8 wk from tumors treated with either IP-10 or HSA. Tumor $n = 12$ at each time-point. (*C*) Representative photograph of A549 tumor-bearing mice treated with either intratumor injection of HSA or IP-10 for 8 wk.

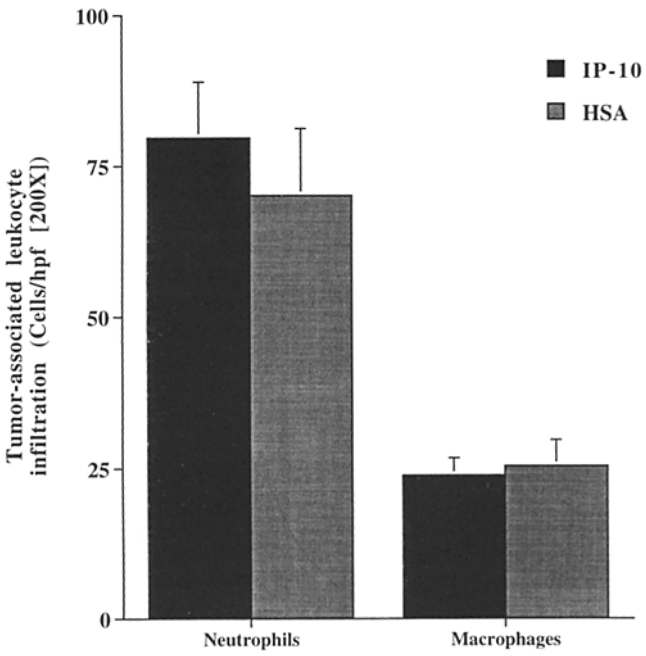

Figure 6. Tumor-associated leukocyte (neutrophil or macrophage) infiltration from A549 tumors treated with either intratumor injection of HSA or IP-10 for 8 wk. Leukocyte infiltration was expressed as the mean number of leukocytes per high power field (200×).

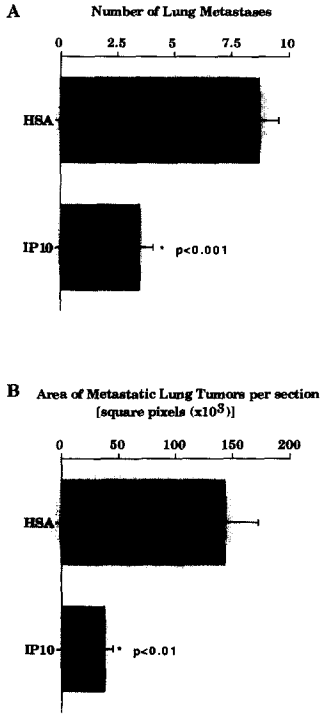

Figure 7. Intratumor injection of IP-10 in primary human adenocarcinoma (A549) tumors in SCID mice inhibits spontaneous lung metastases. (*A*) number of lung metastases in mice treated with either HSA or IP-10 for 8 wk. The number of metastases was expressed per section of lung. (*B*) size of lung metastases in mice treated with either HSA or IP-10 for 8 wk. The size of lung metastases was expressed as square pixels $\times 10^3$.

ization was employed (33, 35). Tumor homogenates were normalized to total protein, incorporated into Hydron pellets, and embedded into the normally avascular rat cornea (Fig. 8). 9 of 12 A549 tumor samples from IP-10 treated tumors induced no significant corneal neovascular response, with the remaining 3 inducing only weak angiogenic activity (Fig. 8, *A–C*). In contrast, 11 of 12 A549 tumor samples from HSA treated tumors induced positive corneal angiogenic responses (Fig. 8, *D–F*). Importantly, there was no infiltration of the corneal tissue by inflammatory cells in any of the test samples, suggesting that the angiogenic responses were mediated entirely by factors present in tumor tissue, rather than by products of infiltrating inflammatory cells. To further confirm that the decreased angiogenic activity correlated with a reduction in tumor vasculature, vessel density by FACS® analysis of Factor VIII-related antigen expressing endothelial cells from the primary tumors was quantified from A549 tumors of SCID mice treated with either intratumor IP-10 or HSA. Tumor-derived Factor VIII-related antigen expressing endothelial cells were markedly reduced in primary tumors treated with IP-10, as compared to HSA (7.8 ± 1.3% vs. 32.4 ± 8.7%, $P < 0.05$, Fig. 9). These studies demonstrated that IP-10 behaved as a potent angiostatic factor for the attenuation of tumor-derived neovascularization leading to reduced tumorigenicity and spontaneous metastases.

Discussion

In this study we demonstrated that IP-10 plays an important role in regulating the angiogenic activity and neovascularization of human NSCLC. IP-10 is a member of the CXC chemokine family (36). In monomeric forms the CXC chemokine family ranges in mass from 7 to 10 kD and are characteristically basic, heparin-binding proteins. These chemokines display four highly conserved cysteine amino acid residues, with the first two cysteines separated by a non-conserved amino acid residue (37, 38). The CXC chemokines are all clustered on human chromosome 4 (q12-q21) and exhibit between 20 to 50% homology on the amino acid level. While the CXC chemokine family was originally recognized for their leukocyte chemotactic activity, these cytokines can also behave as either angiogenic or angiostatic factors depending upon the presence or absence, respectively, of a three amino acid motif, Glu-Leu-Arg (ELR motif) which precedes the first conserved cysteine amino acid residue in the primary sequence (25). For example, platelet factor-4 (PF-4), monokine induced by interferon-gamma (MIG), and IP-10 all lack the ELR motif and are angiostatic factors (25, 39, 40). In contrast, CXC chemokines that contain the ELR motif, such as interleukin-8 (IL-8), neutrophil activating protein-2 (NAP-2), growth-related oncogene alpha (GRO-α), GRO-β, GRO-γ, epithelial neutrophil activating protein-78 (ENA-78), and granulocyte chemotactic protein-2 (GCP-2), induce angiogenic activity at physiological concentrations (25, 41–43).

While the ELR motif appears to be an important structural domain that dictates whether CXC chemokines are angiogenic or angiostatic in their behavior, another characteristic, in general, distinguishes these two groups. The interferons (IFN-α, IFN-β, and IFN-γ) are all known inhibitors of wound repair, especially angiogenesis (18, 44–48).

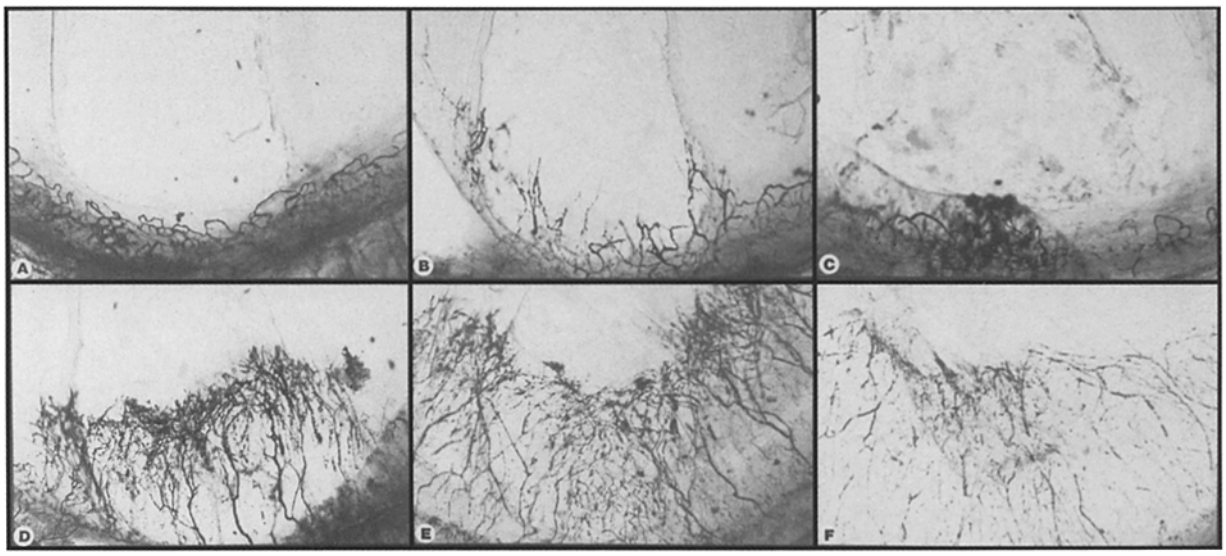

Figure 8. Intratumor injection of IP-10 in primary human adenocarcinoma (A549) tumors in SCID mice reduces their angiogenic activity. *A–C* are representative of the rat corneal angiogenic responses to tumors that had been treated with intratumor IP-10 for 8 wk (25×). *D–F* are representative of the rat corneal angiogenic responses to tumors that had been treated with intratumor HSA for 8 wk (25×).

These cytokines are potent agonists for the expression of IP-10 from a number of cells, including keratinocytes, fibroblasts, endothelial cells, and mononuclear phagocytes (20–23). In contrast, IFN-α, IFN-β, and IFN-γ are all potent inhibitors of the production of monocyte-derived IL-8, GRO-α, and ENA-78 (49, 50), supporting the notion that IFN-α, IFN-β, and IFN-γ may shift the local biological balance of ELR- and non-ELR-CXC chemokines toward a preponderance of angiostatic (IP-10) CXC chemokines. These findings suggest that interferon-γ can alter the balance of expression of angiogenic and angiostatic CXC chemokines which, in the context of tumorigenesis, may be important in regulating net tumor-derived neovascularization.

Angiogenesis is necessary for growth of all solid tumors (5), and the net neovacularization of a tumor is dependent upon an imbalance of the expression of angiogenic and angiostatic factors (51). Therefore, the presence of an angiostatic factor, IP-10, in the context of NSCLC is not unexpected. Other angiostatic factors have been demonstrated to play an important role in the regulation of tumor-associated angiogenesis. O'Reilly and coworkers have described the role of an inhibitor of angiogenesis, angiostatin, in a murine model of lung cancer (12). These investigators found that the production of angiostatin by the primary tumor was not only responsible for inhibition of tumorigenesis within the primary tumor, but its presence in circulation was critical to the suppression of the growth of distant lung metastases. In addition, evidence has linked the downregulation of the local production of angiostatic factors to the pathogenesis and evolution of pre-malignant to malignant disease. This transition to malignant growth has been associated with the activity of the tumor suppressor gene, p53, and its relationship to the angiostatic factor, thrombospondin-1. These studies demonstrated that either loss or mutation of p53 results in a "switch" to a more angiogenic phenotype (11, 14), that coincides with the downregulation and loss of the angiostatic activity attributable to thrombospondin-1 (52). These findings demonstrate the importance of the endogenous expression of angiostatic factors in regulating the biology of malignant tumor growth. Our findings for IP-10 are consistent with the above studies for angiostatin and thrombospondin-1, as levels of IP-10 from both the primary tumor and plasma were inversely correlated with primary tumor growth and spontaneous metastases (A549 adenocarcinoma) to the lung of SCID mice. Moreover, the depletion of IP-10 from Calu 1 (SCCA) tumors resulted in their augmented growth, whereas reconstitution of IP-10 in A549 (adenocarcinomas) tumors reduced their tumorgenicity, tumor-associated neovascularization, and spontaneous metastases to the lung. This effect was attributable to IP-10 inhibition of tumor-derived angiogenesis.

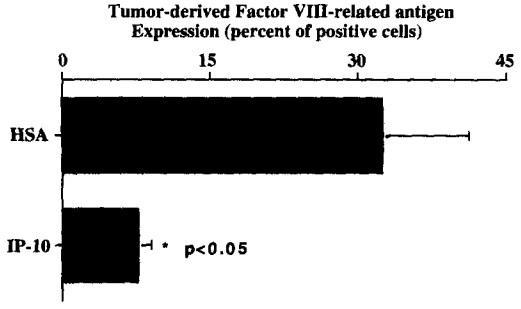

Figure 9. Tumor-associated endothelial cells, as expressed by the percent of Factor VIII-related antigen positive cells by FACS® analysis.

The observation of a marked difference in the levels of IP-10 associated with SCCA, as compared to adenocarcinoma, in freshly isolated NSCLC tumor specimens is pathophysiologically relevant and represents a possible mechanism for the biologic differences of these two cell types of NSCLC. Patient survival and metastatic potential for NSCLC are significantly different. For example, patient survival is lower and metastatic potential is higher for adenocarcinoma, as compared to SCCA of the lung (4, 53). This difference may be due, in part, to the greater IP-10-dependent angiostatic activity found in freshly isolated SCCA tumor specimens. This is supported by the recent findings that SCCA specimens displayed less tumor-derived vasculature than adenocarcinomas of the lung (54). Our finding of higher levels of IP-10 in freshly isolated specimens of SCCA, as compared to adenocarcinoma, demonstrate a potential inverse relationship between IP-10 and tumor vasculature, which may explain the behavior differences of SCCA vs. adenocarinoma. However, to establish a "cause and effect" relationship, we extended these observations and employed a strategy to temporally examine the biology of IP-10 during the growth and metastasis of NSCLC (adenocarcinoma vs. SCCA) in vivo.

The rationale for using these two NSCLC cell lines (A549; adenocarcinoma and Calu 1; squamous cell carcinoma) was based on both their previous use in immunodeficient mice and their heterogeneous characteristics that include: tumorigenicity (A549 >Calu 1) (55); spontaneous lung metastasis (A549 >Calu 1)(55); and experimental lung metastasis (A549 >Calu 1) (55). However, the secretion of metalloproteinases and tissue inhibitors of metalloproteinases (56); expression of integrins (57); or expression of mutants of the tumor suppressor gene, p53 (58) all have failed to correlate with their tumorigenicity and ability to spontaneously metastasize (55–58). We found that the expression of IP-10 from either A549 or Calu 1 tumors inversely correlated with tumor growth in vivo. The nadir of IP-10 in both of these tumor types was associated with a more rapid rate of growth of the primary tumor, as well as the appearance of spontaneous metastases of A549 tumors in the lungs of SCID mice.

To exploit the differences in the expression of IP-10 in A549 and Calu 1 tumors and determine the effect of altering the biology of IP-10 on tumor growth, tumor-associated angiogenic activity and neovascularization, and spontaneous metastases, we used a strategy of either IP-10 depletion in Calu 1 (SCCA) tumors or reconstitution in A549 (adenocarinoma) tumors. Although we did not appreciate any evidence for augmented spontaneous metastases of Calu 1 tumors in SCID mice that had been treated with neutralizing anti-IP-10 antibodies for 10 weeks, we found that the primary tumors were markedly larger in size, as compared to control antibody treated animals. In contrast, intratumor administration of IP-10 significantly reduced the tumor growth and spontaneous metastases of A549 tumors without evidence for augmented recruitment of intratumor leukocytes. This effect was due to reduced tumor-associated angiogenic activity and neovascularization. These results are in agreement with both the previous findings of the anti-tumor effects of intratumor PF-4 (59) or transfection of the gene for IP-10 (60). However, this latter study found that the anti-tumor activity of IP-10 was T cell–dependent, as tumors over-expressing IP-10 failed to regress when engrafted in nude mice (60). In contrast, we found in a model (SCID mice) that is both T and B cell–independent, that intratumor injection of IP-10 into a human NSCLC adenocarcinoma resulted in a striking reduction in tumor-derived angiogenic activity and in cells expressing Factor VIII–related antigen. These effects were unrelated to any difference in tumor infiltrating populations of leukocytes. The difference between these two studies may be related to our use of human NSCLC cell lines as compared to murine tumor cell lines or the use of SCID vs. nude mice. Thus, our finding would support a non-immune function for IP-10 in the inhibition of tumor growth.

In conclusion, tumor-derived angiogenesis is regulated by a dual, yet opposing, balance of angiogenic and angiostatic factors. The magnitude of the expression of angiogenic and angiostatic factors in primary tumors correlates with tumor growth and spontaneous metastases. In this study, we have demonstrated that, in the context of NSCLC, IP-10 is an important endogenous angiostatic factor that regulates tumor growth, tumor-derived angiogenic activity and neovascularization, and potential for spontaneous metastases. These findings support the notion that either reconstitution or augmentation of the expression of IP-10 may be an important treatment strategy for NSCLC.

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Address correspondence to Robert M. Strieter, M.D., Department of Internal Medicine, Division of Pulmonary and Critical Care, University of Michigan Medical Center, 3916 Taubman Center, Box 0360, Ann Arbor, MI 48109-0360.

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