The role of CXC chemokines in the regulation of angiogenesis in non-small cell lung cancer

Douglas A. Arenberg,* Peter J. Polverini,† Steven L. Kunkel,‡ Armen Shanafelt,§ Joseph Hesselgesser,‖ Richard Horuk,‖ and Robert M. Strieter*

*Department of Internal Medicine, Division of Pulmonary and Critical Medicine and ‡Department of Pathology, The University of Michigan Medical School, Ann Arbor; †Laboratory of Molecular Pathology, Department of Oral Medicine, Pathology, and Surgery, The University of Michigan School of Dentistry, Ann Arbor; §Institute of Molecular Biologicals and Institute of Research Technologies, Bayer Corporation, West Haven, Connecticut; and ‖Berlex Biosciences, Division of Berlex Laboratories, Inc., Richmond, California

Abstract: Angiogenesis is a critical component of tumor biology. In recent years newer techniques of cell and molecular biology have led to important advances in our understanding of this process. The regulation of angiogenesis depends on a balance between the activity of local factors that promote (angiogenic factors) or inhibit (angiostatic factors) neovascularization. Nowhere is this paradigm of a balance more apparent than in the study of tumor-associated angiogenesis. Tumors promote angiogenesis through a combination of overexpression of angiogenic factors and local inhibition of angiostatic factors. This strategy leads to an angiogenic environment that promotes tumor growth and metastases. Our laboratory has focused studies on the role of the CXC chemokine family in the regulation of angiogenesis by non-small cell lung cancer (NSCLC). In this article, we review our findings that the CXC chemokine family is composed of members that are either angiogenic or angiostatic. We have found that in NSCLC an imbalance exists in the expression of these factors that favors tumor-derived angiogenesis, and therefore tumor growth and metastases. Furthermore, when this imbalance is corrected to reduce the presence of angiogenic factors or increase the presence of angiostatic factors, tumor growth and metastases are reduced. *J. Leukoc. Biol.* 62: 554–562; 1997.

Key Words: *neovascularization · angiogenic · angiostatic · tumor*

INTRODUCTION

Angiogenesis is an essential biological event [1–7]. Embryonic development, wound healing, chronic inflammation, and the growth of malignant solid tumors represent processes that are strictly dependent on neovascularization. The rate of normal capillary endothelial cell turnover in adults is typically measured in months or years [8, 9]. However, during wound repair, resting endothelial cells become activated, which leads to matrix proteolysis, migration, proliferation, and development of new capillaries within days [1]. An important feature of wound-associated angiogenesis is that it is strictly controlled and transient. As rapidly as neovascularization occurs, these new vessels virtually disappear, returning the tissue vasculature to homeostasis. This abrupt termination of the angiogenic response in the context of resolving wound repair suggests two possible mechanisms of control. First, there is probably a marked reduction in the synthesis and/or release of angiogenic mediators. Second, a simultaneous increase occurs in the levels of angiostatic factors [10]. In contrast to the precise regulation of wound-associated angiogenesis, tumor angiogenesis is characterized by an imbalance favoring over-expression of angiogenic factors and underexpression of angiostatic factors. Several lines of evidence support this contention. First, a salient feature of all solid tumor growth is the presence of neovascularization [3, 5, 10, 11]. Second, in the absence of local capillary proliferation, neoplasms cannot grow beyond the size of 2 mm^3 [12]. Finally, the magnitude of tumor-derived angiogenesis correlates directly with the risk of metastasis of melanoma, prostate cancer, breast cancer, and non-small cell lung cancer (NSCLC) [13–19].

The complement of positive and negative regulators of angiogenesis may vary among different physiological and pathological settings. However, recognition of this dual mechanism of control is critical in order to gain insight

---

Abbreviations: NSCLC, non-small cell lung cancer; PF4, platelet factor 4; PBP, platelet basic protein; CTAP-III, connective tissue activating protein-III; β-TG, β-thromboglobulin; NAP-2, neutrophil activating protein-2; IL-8, interleukin-8; bFGF, basic fibroblast growth factor; HSA, human serum albumin; CMP, corneal micropocket; IP-10, interferon-γ-inducible protein-10; VEGF, vascular endothelial cell growth factor.

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

Received May 9, 1997; revised June 20, 1997; accepted June 22, 1997.

TABLE 1. Representative Examples of Angiogenic and Angiostatic Factors

Angiogenic promoters	Angiogenic inhibitors
Growth factors	Peptides
Acidic fibroblast growth factor (aFGF)	Angiostatin
Basic fibroblast growth factor (bFGF)	Endostatin
Epidermal growth factor (EGF)	Eosinophilic major basic protein
Interleukin-1 (IL-1)	High-molecular-weight hyaluronan
Interleukin-2 (IL-2)	Interferon-α
Scatter factor/hepatocyte growth factor (SF/HGF)	Interferon-β
Transforming growth factor α (TGF-α)	Interferon-γ
Transforming growth factor β (TGF-β)	non-ELR-CXC chemokines
Tumor necrosis factor α (TNF-α)	Interleukin-1
Vascular endothelial growth factor (VEGF)	Interleukin-4
Carbohydrates and lipids	Interleukin-12
12(R)-hydroxyeicosatrienoic acid (Compound D)	Laminin & fibronectin peptides
Hyaluronan fragments	Placental RNase (angiogenin) inhibitor
Lactic acid	Somatostatin
Monobutyrin	Substance P
Prostaglandins E_1 and E_2	Thrombospondin 1
Other proteins and peptides	Tissue inhibitor of metalloproteinases (TIMPs)
Angiogenin	Lipids
Angiotensin II	Angiostatic steroids
Ceruloplasm	Retinoids
Fibrin	Vitamin A
Soluble E-selectin	Others
ELR-CXC chemokines	Nitric oxide
Plasminogen activator	Vitreous fluids
Polyamines	Prostaglandin synthetase inhibitor
Substance P	
Urokinase	
Others	
Adenosine	
Angiotropin	
Copper	
Heparin	
Nicatinamide	
ESAF	

into this complex process and understand the regulation of angiogenesis. The list of factors that can regulate the angiogenic balance is ever-increasing and includes proteins, lipid products, hormones, and other natural or synthetic factors (**Table 1**). In particular, we have found that the CXC chemokine family is composed of potent regulators of angiogenesis [20].

CXC CHEMOKINES

The human CXC chemokine family are cytokines that in their monomeric forms are less than 10 kDa and are characteristically basic heparin-binding proteins (**Table 2**). This family displays four highly conserved cysteine amino acid residues, with the first two cysteines separated by one non-conserved amino acid residue [21–27]. In general, these cytokines appear to have specific chemotactic activity for neutrophils.

Over the last decade several human CXC chemokines have been identified, including platelet factor 4 (PF4), NH_2-terminal truncated forms of platelet basic protein [PBP; connective tissue activating protein-III (CTAP-III), β-thromboglobulin (β-TG), and neutrophil activating protein-2 (NAP-2)], interleukin-8 (IL-8), growth-related oncogene (GRO-α), GRO-β, GRO-γ, interferon-γ-inducible protein-10 (IP-10), monokine induced by interferon-γ (MIG), epithelial neutrophil activating protein-78 (ENA-78), granulocyte chemotactic protein-2 (GCP-2), and stromal cell-derived factor-1 (SDF-1) [21–32]. The NH_2-terminal truncated forms of PBP are generated when this protein is released from platelet α-granules and undergoes proteolytic cleavage by monocyte-derived proteases [33]. PF4 was originally identified for its ability to bind to heparin, leading to inactivation of heparin's anticoagulant function [34]. Both IP-10 and MIG are interferon-inducible chemokines [31, 35–39]. GRO-α, GRO-β, and GRO-γ, are closely related CXC chemokines, with GRO-α originally described for its melanoma growth stimulatory activity [40–42]. IL-8, ENA-78, and GCP-2 were all initially identified on the basis of their ability to induce neutrophil activation and chemotaxis [21–27, 43, 44]. SDF-1 has been recently described for its ability to induce lymphocyte migration and prevent infection of T cells by lymphotropic strains of

TABLE 2. The CXC Chemokines

ELR$^+$
Interleukin-8 (IL-8)
Epithelial neutrophil activating protein-78 (ENA-78)
Growth-related oncogene α (GRO-α, GRO-β, and GRO-γ)
Granulocyte chemotactic protein-2 (GCP-2)
Platelet basic protein (PBP)
Connective tissue activating protein-III (CTAP-III)
β-Thromboglobulin (β-TG)
Neutrophil activating protein-2 (NAP-2)
ELR$^-$
Platelet factor-4 (PF4)
Interferon-γ-inducible protein (IP-10)
Monokine induced by interferon-γ (MIG)
Stromal cell-derived factor-1 (SDF-1)

TABLE 3. NH_2-Terminal Alignment of CXC Chemokines

ELR-CXC chemokines (angiogenic)	
IL-8	S-A-K-E-L-R-C-Q-C
ENA-78	V-L-R-E-L-R-C-V-C
GROα	V-A-T-E-L-R-C-Q-C
GROβ	L-A-T-E-L-R-C-Q-C
GROγ	V-V-T-E-L-R-C-Q-C
Non-ELR-CXC chemokines (angiostatic)	
PF4	E-D-G-***D-L-Q***-C-L-C
IP-10	L-S-R-***T-V-R***-C-T-C
MIG	V-V-R-***K-G-R***-C-S-C
SDF-1	V-S-L-***S-Y-R***-C-P-C

HIV-1 [28–30, 32]. Although numerous investigations have shown the importance of CXC chemokines in acute inflammation as chemotactic/activating factors for neutrophils and mononuclear cells, only recently has it become apparent that these CXC chemokines may be important in the regulation of angiogenesis.

CXC CHEMOKINES AS REGULATORS OF ANGIOGENESIS

Our laboratory and others have previously found that IL-8 can induce angiogenic activity independent of inflammation [45–47]. The angiogenic activity of IL-8 is equivalent on a molar basis to other potent angiogenic factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial cell growth factor (VEGF). IL-8 is a significant angiogenic factor present in freshly isolated human NSCLC [45–48]. It is interesting to note that PF4, another CXC chemokine, is angiostatic [49, 50], and can attenuate tumor growth [51].

Several lines of evidence led our laboratory to speculate that members of the CXC chemokine family may exert disparate effects in mediating angiogenesis as a function of the presence or absence of the ELR motif. First, members of the CXC chemokine family that display binding and activation of neutrophils share the highly conserved ELR motif that immediately precedes the first cysteine amino acid residue, whereas PF4, IP-10, MIG, and SDF-1 lack this motif and do not bind to neutrophils (**Table 3**) [52, 53]. Second, IL-8 contains the ELR motif and is an angiogenic factor [45–47]. In contrast, PF4 lacks the ELR motif and is an angiostatic factor [49, 50], which attenuates growth of tumors in vivo [51]. Third, interferons (IFN-α, IFN-β, and IFN-γ) are inhibitors of wound repair, especially angiogenesis [4, 5, 54, 55], and up-regulate IP-10 and MIG (non-ELR) from a variety of cells [27, 31, 35, 36, 38, 56]. In contrast, we and others have found that IFN-α, IFN-β, and IFN-γ are potent inhibitors of the production of monocyte-derived IL-8, GRO-α, and ENA-78 [57, 58]. These data suggest that interferons may shift the biological balance toward a preponderance of non-ELR CXC chemokines (**Table 4**). If this ELR motif were found to be important in chemokine-mediated angiogenic activity, this would represent a possible mechanism for interferon-dependent inhibition of angiogenesis.

To evaluate this hypothesis, we assessed the endothelial cell chemotactic potential of IL-8, ENA-78, PF4, and IP-10 in varying concentrations. Both IL-8 and ENA-78 demonstrated a dose-dependent increase in endothelial migration that was significantly greater than control [59]. In contrast, neither PF4, IP-10, nor MIG induced significant endothelial cell chemotaxis [59]. We tested other ELR-CXC chemokines for their ability to induce endothelial cell chemotaxis, including GCP-2, GRO-α, -β, and -γ, PBP, CTAP-III, and NAP-2. In a similar fashion to IL-8 or ENA-78, all of the ELR-CXC chemokines induced significant endothelial cell chemotaxis over the background control [59].

We then postulated that the non-ELR CXC chemokines PF4, IP-10, and MIG may be inhibitors of angiogenesis. To test this notion, endothelial cell chemotaxis was performed, as above, in the presence of IL-8, ENA-78, or bFGF with or without varying concentrations of PF4, IP-10, or MIG. In a dose-dependent fashion, the non-ELR CXC chemokines (PF4, IP-10, and MIG) individually inhibited the endothelial cell migration induced by IL-8, ENA-78, or bFGF [59] (**Table 5**).

To determine whether IP-10 or MIG could inhibit in vivo angiogenic activity, the rat corneal micropocket (CMP) assay of neovascularization was used [59]. Cytokine-containing pellets (IL-8, ENA-78, GRO-α, GCP-2, bFGF, or VEGF, either alone or in combination with IP-10 or

TABLE 4. Disparate Regulation of ELR- (IL-8, ENA-78, GRO-α) Versus Non-ELR (IP-10 and MIG)CXC Chemokines by Various Stimuli

	Stimulus			
Chemokines	LPS	TNF	IL-1	IFN-γ
IL-8	++++	++++	++++	----
ENA-78	+++	+++	+++	----
GRO-α	+++	+++	+++	----
IP-10	+	+	+	+++
MIG	−	−	−	+++

TABLE 5. IC_{50} of PF4, IP-10, and MIG for the Inhibition of Endothelial Cell Chemotaxis by the Agonists IL-8, ENA-78, and bFGF

Inhibitor	IL-8 (10 nM)	ENA-78 (10 nM)	bFGF (5 nM)
PF4	5×10^{-11} M	5×10^{-11} M	1×10^{-9} M
IP-10	5×10^{-11} M	5×10^{-11} M	1×10^{-9} M
MIG	5×10^{-10} M	5×10^{-9} M	1×10^{-9} M

MIG) were implanted into the cornea. Neither IP-10 nor MIG induced angiogenic responses in the cornea. However, when combined with the ELR-CXC chemokines (IL-8, ENA-78, GRO-α, or GCP-2), bFGF, or VEGF, IP-10, and MIG significantly inhibited the angiogenic activity of each of these factors. Furthermore, similar to IP-10, MIG and SDF-1 inhibited corneal neovascularization induced by the same factors.

THE ROLE OF THE ELR MOTIF IN CXC CHEMOKINE-INDUCED ANGIOGENESIS

To establish whether the ELR motif is the critical structural/functional domain that dictates angiogenic activity for members of the CXC chemokine family, mutant proteins were constructed by site-directed mutagenesis of IL-8 that contained either TVR (from IP-10) or DLQ (from PF4) amino acid residue substitutions for the ELR motif, and a mutant of MIG was constructed that contained the ELR motif immediately adjacent to the first cysteine amino acid residue of the primary structure of MIG [59]. In endothelial cell chemotaxis assays the TVR-IL-8 or DLQ-IL-8 muteins alone failed to induce endothelial cell chemotactic activity, whereas these muteins inhibited the maximal endothelial chemotactic activity of wild-type IL-8 [59]. Using the in vivo CMP assay of neovascularization, neither the TVR-IL-8 nor the DLQ-IL-8 muteins alone induced a neovascular response. However, both TVR-IL-8 and DLQ-IL-8 muteins inhibited the angiogenic response of either wild-type IL-8 or ENA-78 [59]. Moreover, the angiostatic activity of the TVR-IL-8 and DLQ-IL-8 muteins were not unique to the inhibition of ELR-CXC chemokine-induced angiogenic activity because both IL-8 mutants inhibited bFGF-induced endothelial cell chemotaxis and corneal neovascularization. In addition, when a mutant of MIG was produced containing the ELR motif (ELR-MIG), this molecule induced a significant angiogenic response compared with wild-type MIG [59]. It is interesting to note that wild-type MIG inhibited the angiogenic response of the ELR-MIG mutant in both endothelial migration and CMP assays. Although these studies support the contention that the ELR motif is important in dictating the angiogenic activity of ELR-CXC chemokines, a strategy of scanning mutagenesis was used to investigate the importance of each of the amino acid residues of the ELR motif in angiogenesis. Substitution of the amino acid residue (arginine → alanine) of the ELR motif of wild-type GRO-α completely attenuated its ability to induce angiogenic activity in the CMP assay. Furthermore, this mutant inhibited the angiogenic activity of wild-type GRO-α, as well as bFGF and VEGF. These data further support the importance of the ELR motif, specifically the arginine amino acid residue, as a structural domain that dictates the angiogenic activity of these CXC chemokines.

POTENTIAL ENDOTHELIAL RECEPTORS FOR CXC CHEMOKINE-MEDIATED CONTROL OF ANGIOGENESIS

Luster and colleagues have found that IP-10 binds to a specific cell surface site on endothelial cells that is shared by PF-4. This receptor appears to be a heparan sulfate proteoglycan because binding could be inhibited by heparanase pretreatment of the endothelial cells [60]. This binding site is specific for IP-10 and PF4 because neither ELR containing CXC chemokines nor various CC chemokines compete for binding on endothelial cells. Furthermore, these investigators found that binding of IP-10 to endothelial cells inhibited proliferation. This inhibition was independent of calcium flux and apoptosis, and dependent on reversible cell cycle arrest. Although it is not clear that this receptor represents the recently identified receptors for IP-10/MIG (CXCR3) or SDF-1 (CXCR4) expressed on T cells [29, 32, 61], these findings suggest that IP-10, PF4, and potentially MIG and SDF-1 may share a heparan sulfate proteoglycan component of their receptor that accounts for their binding to endothelial cells and subsequent angiostatic activity.

In contrast to the recently described specific proteoglycan receptor for IP-10 and PF4 on endothelial cells, a specific endothelial receptor(s) has not been established for the activity of ELR-CXC chemokine-induced angiogenesis. However, evidence would suggest that the endothelial receptor for ELR-CXC chemokines is the CXC chemokine receptor, CXCR2. In support of this contention are the following. (1) Although expression of mRNA for IL-8 receptor A (CXCR1) has been identified in endothelial cells by reverse transcriptase-polymerase chain reaction, this same study found that IL-8 and NAP-2 could compete for binding on endothelial cells [62]. However, CXCR1 binds only IL-8 and not NAP-2 [63]. (2) CXCR2 on neutrophils binds all ELR-CXC chemokines [63, 64], and all ELR-CXC chemokines are angiogenic [59]. (3) Although the Duffy antigen receptor for chemokines (DARC) has been identified on post-capillary venule endothelial cells [65], this receptor binds not only ELR-CXC chemokines, but also CC chemokines, which to date have not been implicated in the control of angiogenesis [66]. (4) Healing human burn wounds express CXCR2 in association with capillary endothelial cells in areas of neovascularization [67]. Nevertheless, further studies will be required to delineate the

specific endothelial cell receptor(s) for the angiogenic activities of the ELR-CXC chemokines.

IL-8 PROMOTES NEOVASCULARIZATION IN LUNG CANCER

Our laboratory originally described the presence of elevated levels of IL-8 in NSCLC and determined that IL-8 significantly contributed to overall tumor-derived angiogenic activity [48]. In this study, IL-8 accounted for 42–80% of the angiogenic activity for each of the tumor specimens, as determined by bioassays of angiogenesis [48]. The relative contribution of IL-8 to tumor-derived angiogenic activity was compared with other known angiogenic factors in NSCLC. Neutralizing antibodies to IL-8 resulted in a significant reduction of endothelial cell chemotactic activity in response to NSCLC tissue, with a decline to 75, 39, and 61% of the standard bioactivity for adenocarcinoma and squamous carcinoma tissue samples, and A549 (adenocarcinoma) conditioned media, respectively [48]. In contrast, anti-bFGF antibodies had no significant effect on the endothelial cell chemotaxis in response to samples of A549 (adenocarcinoma) cells or squamous cell carcinoma tissue. However, anti-bFGF neutralizing antibodies reduced the endothelial cell chemotactic activity from adenocarcinoma tissue by 35% [48]. It is interesting that the neutralization of transforming growth factor-α (TGF-α) had no significant effect on endothelial cell chemotaxis in response to adenocarcinoma or to the A549 cell line. However, these antibodies resulted in a significant reduction in the endothelial cell chemotactic response to squamous cell carcinoma tissue [48]. Although bFGF and TGF-α have been previously described as potential tumor-angiogenic factors, our studies were the first to demonstrate that a primary angiogenic signal for NSCLC neovascularization was directly mediated by tumor-associated IL-8.

To extend the above studies to an in vivo model system of human tumorigenesis, we employed a human NSCLC/SCID mouse chimera by injecting either the human NSCLC cell lines A549 (adenocarcinoma) or Calu 1 (squamous cell carcinoma) into SCID mice [68]. 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. The production of IL-8 from A549 tumors increased in direct correlation with tumor size. In contrast, the production of IL-8 by Calu 1 tumors was delayed, yet still correlated with tumor size (**Fig. 1**). A549 (adenocarcinoma) tumors produced markedly greater levels of IL-8 and were 50-fold larger in size than Calu 1 (squamous cell carcinoma) tumors by 8 weeks.

To delineate whether IL-8 contributed to tumorigenesis of A549 cells in SCID mice, A549 (adenocarcinoma) tumor-bearing animals were passively immunized with neutralizing IL-8 antibodies, control antibodies, or were left untreated. A549 tumor-bearing animals treated with neutralizing antibodies to IL-8 demonstrated a >40% reduction in tumor growth compared with animals that were either untreated or treated with control antibodies [68]. There was no difference in the leukocyte infiltration of the tumors treated with anti-IL-8 or control antibody. SCID mice treated with neutralizing antibodies to IL-8 demonstrated a reduction in the number of metastatic cells to the lung compared with control antibody-treated animals [68].

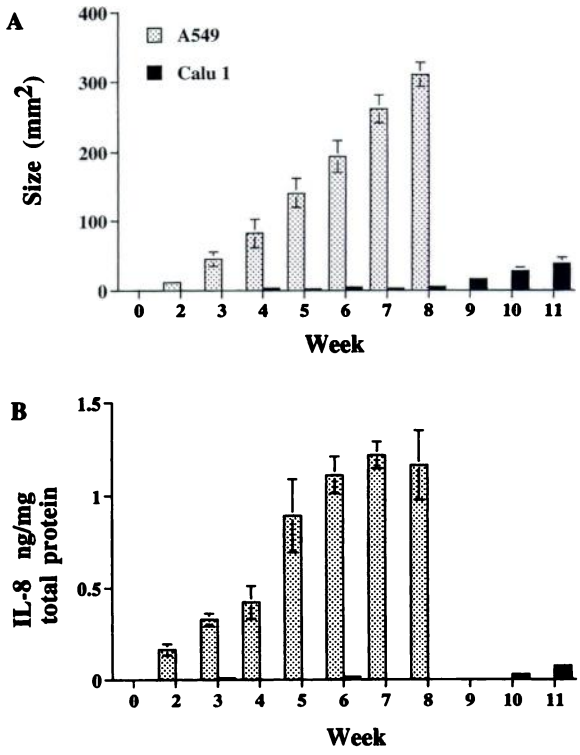

Fig. 1. A549 and Calu1 NSCLC tumor growth and IL-8 production in SCID mice. (A) Time course of A549 (adenocarcinoma) and Calu 1 (squamous cell carcinoma) tumor growth. (B) Time course of IL-8 expression (ng IL-8/mg total protein) in these tumors. Both Calu 1 ($r = 0.87$) and A549 ($r = 0.95$) tumor growth correlated highly with IL-8 expression.

To further determine the mechanism of tumor growth inhibition, ex vivo angiogenic activity was evaluated from A549 tumors of animals that had been treated in vivo with either control or neutralizing IL-8 antibodies. A549 tumor-derived angiogenic activity, as assessed in the corneal micropocket assay, was significantly reduced in tumors from mice treated with anti-IL-8 compared with controls. To further confirm that decreased angiogenic activity correlated with a reduction in tumor vascularity, vessel density was quantified from A549 tumors of SCID mice treated with either control or neutralizing IL-8 antibodies. Tumor vessel density in animals treated with neutralizing IL-8 antibodies was significantly lower than in tumors of animals treated with control antibody. These studies demonstrated that a primary angiogenic signal for A549 (adenocarcinoma) tumor angiogenesis in vivo was directly mediated by tumor-associated IL-8.

IP-10 IS AN ENDOGENOUS ANGIOSTATIC IN NSCLC

To determine whether IP-10 protein was present in human NSCLC in a similar manner as IL-8 above, freshly isolated specimens of human NSCLC tumors were assessed by specific IP-10 enzyme-linked immunosorbent assay [69]. The levels of IP-10 from tumor specimens were actually higher than in normal lung tissue. To ascertain whether expression of IP-10 protein varied by histological tumor type, results were further subdivided by cell type (squamous cell carcinoma vs. adenocarcinoma). The increase in IP-10 from NSCLC tissue was entirely attributable to the higher levels of IP-10 present in squamous cell carcinoma compared with adenocarcinoma. The observation of a marked difference in the levels of IP-10 associated with squamous cell carcinoma compared with adenocarcinoma is pathophysiologically relevant and represents a possible mechanism for the biological differences of these two cell types of NSCLC. Patient survival and metastatic potential for NSCLC are significantly different for these two cell types. For example, patient survival is poorer and metastatic potential is greater for adenocarcinoma compared with squamous cell carcinoma of the lung [70, 71]. This difference may be due, in part, to the greater IP-10-dependent angiostatic activity found in squamous cell carcinoma tumor specimens. This is supported by the recent findings that squamous cell carcinoma displays less vessel density than adenocarcinoma of the lung [72]. The finding of higher levels of IP-10 in freshly isolated specimens of squamous cell carcinoma compared with adenocarcinoma demonstrates a potential inverse relationship between IP-10 and tumor vascularity, which may explain the behavioral differences between these two cell types of NSCLC.

Although these experiments demonstrated that IP-10 levels were significantly elevated in specimens of freshly isolated squamous cell carcinoma, we postulated that IP-10 may be acting in vivo to regulate tumor-derived angiogenesis [69]. To test this hypothesis, we preincubated specimens of human squamous cell carcinoma in the presence of either control or neutralizing antibodies to IP-10 and assessed their angiogenic activity using either in vitro endothelial cell chemotaxis or CMP assays. Inhibition of IP-10 in samples of squamous cell carcinoma led to a significant increase in endothelial cell chemotactic activity. These findings were further confirmed using CMP assay, as squamous cell carcinoma specimens preincubated with neutralizing antibodies to IP-10, as compared to control antibodies, demonstrated augmented neovascularization in the cornea.

The above findings suggested that IP-10 represented an important endogenous angiostatic factor in squamous cell carcinoma of the lung. However, to determine whether this angiostatic activity was physiologically relevant during the course of in vivo tumor growth, the human NSCLC/SCID mouse model of tumorigenesis was employed. SCID mice were inoculated with A549 (adenocarcinoma) or Calu 1 (squamous cell carcinoma) cells similar to the experiments outlined above for assessing IL-8 [68, 69]. However, in contrast to IL-8, we found the opposite phenomenon for IP-10. The production of IP-10 in both A549 and Calu 1 tumors was inversely correlated with tumor growth. However, IP-10 levels were significantly higher in the Calu 1 (squamous cell carcinoma) tumors compared with A549 (adenocarcinoma) tumors (**Fig. 2**). Plasma levels of IP-10 paralleled those found in the primary tumors. Furthermore, the appearance of spontaneous lung metastases in SCID mice bearing A549 tumors occurred when IP-10 levels (primary tumor and plasma) reached a nadir. To determine whether IP-10 in vitro inhibited the proliferation of these cell lines, A549 and Calu 1 cells were cultured in the presence or absence of recombinant IP-10. The presence of exogenous IP-10 did not alter proliferation compared with appropriate controls. These findings suggested that IP-10 neither functions as an autocrine growth factor nor an inhibitor of cellular proliferation of human NSCLC cell lines.

Because IP-10 was found to be a potent endogenous angiostatic molecule in squamous cell carcinoma, the reduced expression of IP-10 in A549 (adenocarcinoma) tumors, as compared to Calu 1 (squamous cell carcinoma) tumors, may contribute to their more aggressive behavior. We hypothesized that restoration of tumor-associated IP-10 in A549 tumors would lead to inhibition of tumorigenesis via an IP-10-dependent decrease in angiogenic activity. SCID mice bearing A549 tumors were injected with re-

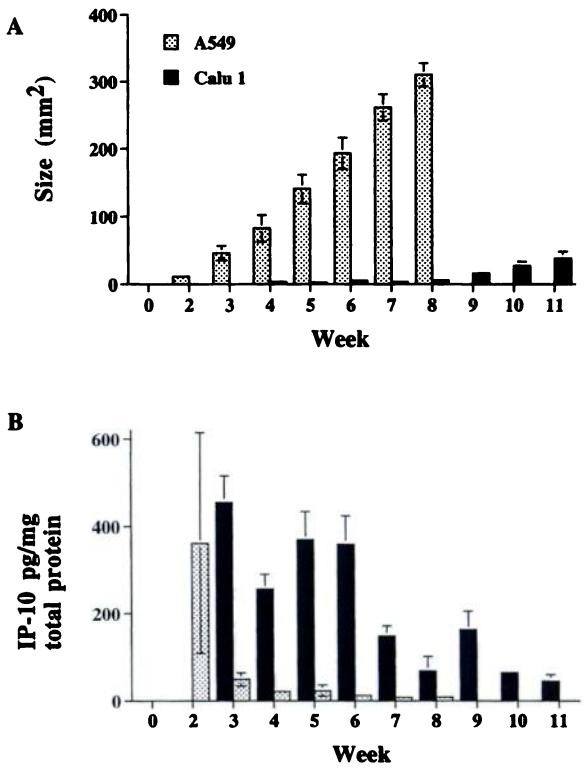

Fig. 2. In vivo growth rate (A) and IP-10 expression (pg/mg total protein, B) of A549 and Calu 1 tumors. In contrast to IL-8, IP-10 expression by these tumors correlates inversely with tumor growth.

combinant human IP-10 (1 μg intratumor) or an equimolar concentration of an irrelevant human protein (human serum albumin, HSA), every 48 h for a period of 8 weeks 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. To exclude that IP-10 inhibited tumor growth by recruiting tumoricidal leukocytes, quantitation of tumor-infiltrating leukocytes was performed by immunohistochemistry. A549 tumors from IP-10-treated SCID mice revealed no evidence for increased leukocyte infiltration compared with control tumors.

To determine the mechanism of growth inhibition by intratumor administration of IP-10, we directly evaluated angiogenic activity in the CMP assay from A549 tumors of animals that had been treated in vivo with IP-10. Nine of 12 A549 tumor samples from IP-10-treated tumors induced no significant neovascular response, with the remaining three inducing only weak angiogenic activity. In contrast, 11 of 12 A549 tumor samples from control-treated tumors induced positive angiogenic responses. To confirm that the decreased angiogenic activity correlated with a reduction in tumor vascularity, vessel density was assessed by FACS analysis of Factor VIII-related antigen expressing endothelial cells from the primary tumor. Tumor-derived Factor VIII-related antigen expressing endothelial cells were markedly reduced in primary tumors treated with IP-10, supporting the notion that IP-10 reduced the neovascularization of the experimental tumors, and is a potent endogenous angiostatic factor in NSCLC.

Lung sections from tumor-bearing SCID mice treated with either intratumor IP-10 or HSA were examined for evidence of spontaneous metastases [69]. The number of metastases was significantly reduced in mice treated with IP-10. In addition, the size (area) of the lung metastases per section was also dramatically reduced in the IP-10-treated group.

To further demonstrate the importance of endogenous IP-10 in the regulation of human NSCLC (squamous cell carcinoma) tumor growth, we passively immunized SCID mice bearing Calu 1 tumors with either neutralizing rabbit anti-human IP-10 or control antibodies. Recall that Calu 1 tumors expressed higher levels of IP-10 during tumor growth and were slower growing when compared with A549 (adenocarcinoma) tumors. Calu 1 tumors from animals that were passively immunized with neutralizing antibodies to IP-10 demonstrated a 1.8- to 2.9-fold increase in tumor size compared with animals that had received control antibodies. Therefore, the presence of increased levels of IP-10 in Calu 1 (squamous cell) tumors relative to A549 (adenocarcinoma) tumors appears to contribute to differences in their behavior in SCID mice.

CONCLUSION

Angiogenesis is regulated by a dual, yet opposing, system of angiogenic and angiostatic factors. The above studies using both in vitro and in vivo systems have demonstrated that, as a family, the CXC chemokines behave as either angiogenic or angiostatic factors, depending on the presence of the ELR motif. In the context of NSCLC and perhaps other solid tumors, CXC chemokines are important endogenous factors that regulate tumor growth, tumor-derived angiogenic activity, and potential for spontaneous metastases. These findings provide new insights into the biology of NSCLC, and present an opportunity to target new therapies.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants CA72543 (to D. A. A.), CA66180, P50 HL56402, P50 CA69568 (to R. M. S.), HL39926 (to P. J. P.), HL31693, and HL35276 (S. L. K.).

REFERENCES

1. Leibovich, S. J., Weisman, D. M. (1988) Macrophages, wound repair and angiogenesis. *Prog. Clin. Biol. Res.* **266**, 131–145.
2. Auerbach, R. (1981) *Angiogenesis-Inducing Factors: a Review.* New York: Academic Press.
3. Folkman, J., Cotran, R. (1976) Relation of vascular proliferation to tumor growth. *Int. Rev. Exp. Pathol.* **16**, 207–248.
4. Folkman, J., Klagsbrun, M. (1987) Angiogenic factors. *Science* **235**, 442–447.
5. Folkman, J. (1993) Tumor angiogenesis. In *Cancer Medicine, Vol. 1* (J. F. Holland, Frei III, E., Bast, R. C., Kufe, D. W., D. L. Morton, and R. R. Weischelbaum, eds.), Philadelphia, PA: Lea & Febiger, 153–170.
6. Folkman, J. (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* **1**, 27–31.
7. Polverini, P. J., Cotran, P. S., Gimbrone, M. A., Unanue, E. R. (1977) Activated macrophages induce vascular proliferation. *Nature* **269**, 804–806.
8. Engerman, R. L., Pfaffenenbach, D., Davis, M. D. (1967) Cell turnover of capillaries. *Lab. Invest.* **17**, 738–743.
9. Tannock, I. F., Hayashi, H. S. (1972) The proliferation of capillary and endothelial cells. *Cancer Res.* **32**, 77–82.
10. Bouck, N. (1992) Angiogenesis: a mechanism by which oncogenes and tumor suppressor genes regulate tumorigenesis. *Cancer Treat. Res.* **63**, 359–371.
11. Bouck, N. (1990) Tumor angiogenesis: the role of oncogenes and tumor suppressor genes. *Cancer Cells* **2**, 179–185.
12. Gimbrone, M. A., Leapman, S. B., Cotran, R. S., Folkman, J. (1972) Tumor dormancy in vivo by prevention of neovascularization. *J. Exp. Med.* **136**, 261–276.
13. Folkman, J., Watson, K., Ingber, D., Hanahan, D. (1989) Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature* **339**, 58–61.
14. Maiorana, A., Gullino, P. M. (1978) Acquisition of angiogenic capacity and neoplastic transformation in the rat mammary gland. *Cancer Res.* **38**, 4409–4414.
15. Herlyn, M., Clark, W. H., Rodeck, U., Manciati, M. L., Jambrosic, J., Koprowski, H. (1987) Biology of tumor progression in human melanocytes. *Lab. Invest.* **56**, 461–474.
16. Weidner, N. (1995) Intratumor microvessel density as a prognostic factorin cancer. *Am. J. Pathol.* **147**, 9–19.
17. Weidner, N., Carroll, P. R., Flax, J., Blumenfeld, W., Folkman, J. (1993) Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am. J. Pathol.* **143**, 401–409.
18. Weidner, N., Semple, J. P., Welch, W. R., Folkman, J. (1991) Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. *N. Engl. J. Med.* **324**, 1–8.
19. Macchiarini, P., Fontanini, G., Hardin, M. J., Squartini, F., Angeletti, C. A. (1992) Relation of neovascularization to metastasis of non-small cell lung cancer. *Lancet* **340**, 145–146.
20. Strieter, R. M., Polverini, P. J., Kunkel, S. L., Arenberg, D. A., Burdick,

M. D., Kasper, J., Dzuiba, J., Damme, J. V., Walz, A., Marriott, D., Chan, S. Y., Roczniak, S., Shanafelt, A. B. (1995) The functional role of the 'ELR' motif in CXC chemokine-mediated angiogenesis. *J. Biol. Chem.* **270**, 27348–27357.

21. Walz, A., Kunkel, S. L., Strieter, R. M. (1996) CXC chemokines–an overview. In *Chemokines in Disease* (A. E. Koch and R. M. Strieter, eds.), Austin, TX: Landes, 1–26.
22. Strieter, R. M., Kunkel, S. L. (1997) Chemokines and the lung. In *Lung: Scientific Foundations*, 2nd ed. (R. Crystal, J. West, E. Weibel, and P. Barnes, eds.), New York: Raven Press, 155–186.
23. Strieter, R. M., Lukacs, N. W., Standiford, T. J., Kunkel, S. L. (1993) Cytokines and lung inflammation. *Thorax* **48**, 765–769.
24. Baggiolini, M., Walz, A., Kunkel, S. L. (1989) Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *J. Clin. Invest.* **84**, 1045–1049.
25. Baggiolini, M., Dewald, B., Walz, A. (1992) Interleukin-8 and related chemotactic cytokines. In *Inflammation: Basic Principles and Clinical Correlates* (J. I. Gallin, I. M. Goldstein, and R. Snyderman, eds.), New York: Raven Press.
26. Matsushima, K., Oppenheim, J. J. (1989) Interleukin 8 and MCAF: Novel inflammatory cytokines inducible by IL-1 and TNF. *Cytokine* **1**, 2–13.
27. Miller, M. D., Krangel, M. S. (1992) Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines. *Crit. Rev. Immunol.* **12**, 17–46.
28. Tashiro, K., Tada, H., Heilker, R., Shirozu, M., Nakano, T., Honjo, T. (1993) Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. *Science* **261**, 600–603.
29. Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., Springer, T. A. (1996) The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* **382**, 829–833.
30. Shirozu, M., Nakano, T., Inazawa, J., Tashiro, K., Tada, H., Shinohara, T., Honjo, T. (1995) Structure and chromosomal localization of the human stromal cell-derived factor 1 (SDF-1) gene. *Genomics* **28**, 495–500.
31. Farber, J. M. (1993) HuMIG: a new member of the chemokine family of cytokines. *Biochem. Biophys. Res. Commun.* **192**, 223–230.
32. Oberlin, E., Amara, A., Bachelerie, F., Bessia, C., Virelizier, J.-L., Arenzana-Seisdedos, F., Schwartz, O., Heard, J.-M., Clark-Lewis, I., Legler, D. F., Loetscher, M., Baggiolini, M., Moser, B. (1996) The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature* **382**, 833–835.
33. Walz, A., Baggiolini, M. (1990) Generation of the neutrophil-activating peptide NAP-2 from platelet basic protein or connective tissue-activating peptide III through monocyte proteases. *Exp. Med.* **171**, 449–454.
34. Deutsch, E., Kain, W. (1961) Studies on platelet factor 4. In *Blood platelets* (S. A. Jonson, R. W. Monto, J. W. Rebuck, and R. C. Horn, eds.), Boston: Little-Brown.
35. Farber, J. M. (1990) A macrophage mRNA selectively induced by gamma-interferon encodes a member of the platelet factor 4 family of cytokines. *Proc. Natl. Acad. Sci. USA* **87**, 5238–5242.
36. Farber, J. M. (1992) A collection of mRNA species that are inducible in the RAW 264.7 mouse macrophage cell line by gamma interferon and other agents. *Mol. Cell. Biol.* **12**, 1535–1545.
37. Luster, A. D., Unkeless, J. C., Ravetch, J. V. (1985) Gamma-interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. *Nature* **315**, 672–676.
38. Luster, A. D., Ravetch, J. V. (1987) Biochemical characterization of a gamma interferon-inducible cytokine (IP-10). *J. Exp. Med.* **166**, 1084–1097.
39. Luster, A. D., Jhanwar, S. C., Chaganti, R. S., Kersey, J. H., Ravetch, J. V. (1987) Interferon-inducible gene maps to a chromosomal band associated with a (4;11) translocation in acute leukemia cells. *Proc. Natl. Acad. Sci. USA* **84**, 2868–2871.
40. Ansiowicz, A., Bardwell, L., Sager, R. (1987) Constitutive overexpression of a growth-regulated gene in transformed Chinese hamster and human cells. *Proc. Natl. Acad. Sci. USA* **84**, 7188–7192.
41. Ansiowicz, A., Zajchowski, D., Stenman, G., Sager, R. (1988) Functional diversity of gro gene expression in human fibroblasts and mammary epithelial cells. *Proc. Natl. Acad. Sci. USA* **85**, 9645–9649.
42. Richmond, A., Thomas, H. G. (1988) Melanoma growth stimulatory activity: isolation from human melanoma tumors and characterization of tissue distribution. *J. Cell Biochem.* **36**, 185–198.
43. Oppenheim, J. J., Zachariae, O. C., Mukaida, N., Matsushima, K. (1991) Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu. Rev. Immunol.* **9**, 617–648.
44. Walz, A., Burgener, R., Car, B., Baggiolini, M., Kunkel, S. L., Strieter, R. M. (1991) Structure and neutrophil-activating properties of a novel inflammatory peptide (ENA-78) with homology to interleukin-8. *J. Exp. Med.* **174**, 1355–1362.
45. Koch, A. E., Polverini, P. J., Kunkel, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M., Elner, S. G., Strieter, R. M. (1992) Interleukin-8 (IL-8) as a macrophage-derived mediator of angiogenesis. *Science* **258**, 1798–1801.
46. Hu, D. E., Hori, Y., Fan, T. P. D. (1993) Interleukin-8 stimulates angiogenesis in rats. *Inflammation* **17**, 135–143.
47. Strieter, R. M., Kunkel, S. L., Elner, V. M., Martonyl, C. L., Koch, A. E., Polverini, P. J., Elner, S. G. (1992) Interleukin-8: A corneal factor that induces neovascularization. *Am. J. Pathol.* **141**, 1279–1284.
48. Smith, D. R., Polverini, P. J., Kunkel, S. L., Orringer, M. B., Whyte, R. I., Burdick, M. D., Wilke, C. A., Strieter, R. M. (1994) Inhibition of IL-8 attenuates angiogenesis in bronchogenic carcinoma. *J. Exp. Med.* **179**, 1409–1415.
49. Maione, T. E., Gray, G. S., Petro, J., Hunt, A. J., Donner, A. L., Bauer, S. I., Carson, H. F., Sharpe, R. J. (1990) Inhibition of angiogenesis by recombinant human platelet factor-4. *Science* **247**, 77–79.
50. Maione, T. E., Gray, G. S., Hunt, A. J., Sharpe, R. J. (1991) Inhibition of tumor growth in mice by an analogue of platelet factor 4 that lacks affinity for heparin and retains potent angiostatic activity. *Cancer Res.* **51**, 2077–2083.
51. Sharpe, R. J., Byers, H. R., Scott, C. F., Bauer, S. I., Maione, T. E. (1990) Growth inhibition of murine melanoma and human colon carcinoma by recombinant human platelet factor 4. *J. Natl. Cancer Inst.* **82**, 848–853.
52. Clark-Lewis, I., Dewald, B., Geiser, T., Moser, B., Baggiolini, M. (1993) Platelet factor 4 binds to interleukin 8 receptors and activates neutrophils when its N terminus is modified with Glu-Leu-Arg. *Proc. Natl. Acad. Sci. USA* **90**, 3574–3577.
53. Hebert, C. A., Vitangcol, R. V., Baker, J. B. (1991) Scanning mutagenesis of interleukin-8 identifies a cluster of residues required for receptor binding. *J. Biol. Chem.* **266**, 18989–18994.
54. Folkman, J., Brem, H. (1992) Angiogenesis and inflammation. In *Inflammation: Basic Principles and Clinical Correlates.* 2nd ed. (J. I. Gallin, I. M. Goldstein, and R. Snyderman, eds.), New York: Raven Press, 821–839.
55. Klagsbrun, M., D'Amore, P. A. (1991) Regulators of angiogenesis. *Annu. Rev. Physiol.* **53**, 217–239.
56. Luster, A. D., Weinshank, R. L., Feinman, R., Ravetch, J. V. (1988) Molecular and biochemical characterization of a novel gamma-interferon-inducible protein. *J. Biol. Chem.* **263**, 12036–12043.
57. Gusella, G. L., Musso, T., Bosco, M. C., Espinoza-Delgado, I., Matsushima, K., Varesio, L. (1993) IL-2 up-regulates but IFN-γ suppresses IL-8 expression in human monocytes. *J. Immunol.* **151**, 2725–2732.
58. Schnyder-Candrian, S., Strieter, R. M., Kunkel, S. L., Walz, A. (1995) Interferon-α and interferon-γ downregulate the production of interleukin-8 and ENA-78 in human monocytes. *J. Leukoc. Biol.* **57**, 929–935.
59. Strieter, R. M., Polverini, P. J., Kunkel, S. L., Arenberg, D. A., Burdick, M. D., Kasper, J., Dzuiba, J., Van Damme, J., Walz, A., Marriott, D., Chan, S., Roczniak, S., Shanafelt, A. (1995) The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J. Biol. Chem.* **270**, 27348–27357.
60. Luster, A. D., Greenberg, S. M., Leder, P. (1995) The IP-10 chemokine binds to a specific cell surface heparan sulfate shared with platelet factor 4 and inhibits endothelial cell proliferation. *J. Exp. Med.* **182**, 219–232.
61. Loetscher, M., Gerber, B., Loetscher, P., Jones, S. A., Piali, L., Clark-Lewis, I., Baggiolini, M., Moser, B. (1996) Chemokine receptor specific for IP-10 and Mig: structure, function, and expression in activated T-lymphocytes. *J. Exp. Med.* **184**, 963–969.
62. Schönbeck, U., Brandt, E., Petersen, F., Flad, H., Loppnow, H. (1995) IL-8 specifically binds to endothelial but not to smooth muscle cells. *J. Immunol.* **154**, 2375–2383.
63. Lee, J., Horuk, R., Rice, G. C., Bennett, G. L., Camerato, T., Wood, W. I. (1992) Characterization of two high affinity human interleukin-8 receptors. *J. Biol. Chem.* **267**, 16283–16287.
64. Taub, D. D., Oppenheim, J. J. (1994) Chemokines, inflammation and immune system. *Ther. Immunol.* **1**, 229–246.
65. Hadley, T. J., Lu, Z., Wasniowska, K., Peiper, S. C., Hesselgesser, J., Horuk, R. (1994) Postcapillary venule endothelial cells in kidney express a multispecific chemokine receptor that is structurally and functionally identical to the erythroid isoform, which is the Duffy bloodgroup antigen. *J. Clin. Invest.* **94**, 985–991.
66. Neote, K., Darbonne, W., Ogez, J., Horuk, R., Schall, T. (1993) Identification of a promiscuous inflammatory peptide receptor on the surface of red blood cells. *J. Biol. Chem.* **268**, 985–991.
67. Nanney, L. B., Mueller, S. G., Bueno, R., Peiper, S. C., Richmond, A. (1995) Distributions of melanoma growth stimulatory activity or growth-related gene and the interleukin-8 receptor type B in human wound repair. *Am. J. Pathol.* **147**, 1248–1260.
68. Arenberg, D. A., Kunkel, S. L., Polverini, P. J., Glass, M., Burdick, M. D., Strieter, R. M. (1996) Inhibition of interleukin-8 reduces tumorigenesis of human non-small cell lung cancer in SCID mice. *J. Clin. Invest.* **97**, 2792–2802.
69. Arenberg, D. A., Kunkel, S. L., Polverini, P. J., Morris, S. B., Burdick, M. D.,

Glass, M., Taub, D. T., Iannetoni, M. D., Whyte, R. I., Strieter, R. M. (1996) Interferon-γ-inducible protein 10 (IP-10) is an angiostatic factor that inhibits human non-small cell lung cancer (NSCLC) tumorigenesis and spontaneous metastases. *J. Exp. Med.* **184**, 981–992.

70. Carney, D. N. (1988) Cancers of the lungs. In *Pulmonary Diseases and Disorders* (A. P. Fishman, ed.), New York: McGraw-Hill, 1885–2068.

71. Minna, J. D. (1991) Neoplasms of the lung. In *Principles of Internal Medicine* (K. J. Isselbacher, ed.), New York: McGraw-Hill, 1102–1110.

72. Yuan, A., Pan-Chyr, Y., Chong-Jen, Y., Lee, Y., Yu-Tuang, Y., Chi-Long, C., Lee, L., Sow-Hsong, K., Kwen-Tay, L. (1995) Tumor angiogenesis correlates with histologic type and metastasis in non-small cell lung cancer. *Am. J. Respir. Crit. Care Med.* **152**, 2157–2162.