Role of C-X-C chemokines as regulators of angiogenesis in lung cancer

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Abstract: Lung cancer is the leading cause of malignancy-related mortality in the U.S. and is predicted to increase over the remainder of this decade. Despite attempts to advance early diagnosis and use combination therapies, the clinical response of this cancer yields an overall 5-year survival rate of less than 15%. Clearly, new strategies for therapy are indicated. Although carcinogenesis is complex, tumor growth beyond 1–2 mm^3 is dependent on angiogenesis. One of the potential mechanisms that allows for tumorigenesis is dysregulation of the balance of angiogenic and angiostatic factors that favors net neovascularization within the primary tumor. Numerous studies have investigated the role of a variety of molecules in the regulation of angiogenesis. Recently, interleukin-8 (IL-8), a member of the C-X-C chemokine family, has been found to be an angiogenic factor. In contrast, platelet factor 4 (PF4), another C-X-C chemokine, has been shown to have angiostatic properties. It is interesting that the major structural difference between IL-8 and PF4 is the presence of the NH_2-terminal ELR (Glu-Leu-Arg) motif that precedes the first cysteine amino acid residue of IL-8 and is important in ligand/receptor interactions. We hypothesize that angiogenesis associated with tumorigenesis is dependent on members of the C-X-C chemokine family acting as either angiogenic or angiostatic factors. This paradigm predicts that the biological balance in the expression of these C-X-C chemokines dictates whether the neoplasm grows and develops metastatic potential or regresses. In this review we discuss our recent laboratory findings that support this contention and suggest that further elucidation of the biology of C-X-C chemokines in the context of neovascularization of nonsmall cell lung cancer will permit novel targeted therapy aimed specifically at attenuating tumor growth and metastasis. *J. Leukoc. Biol.* 57: 752–762; 1995.

Key Words: *tumor genesis • cytokines • neurovascularization • chemotaxis*

INTRODUCTION

Lung cancer is the leading cause of malignancy-related mortality in the U.S. [1]. Although the incidence of several other malignancies has declined or remained stable, the occurrence of bronchogenic carcinoma has escalated to near-epidemic proportions. Over 150,000 new cases are diagnosed and an equal number of deaths annually are attributable to bronchogenic carcinoma in the U.S. [1]. Despite attempts to advance early diagnosis and use combination therapies, the clinical response of this tumor yields an overall 5-year survival rate for lung cancer patients of less than 15% [2]. Although lung cancer prevention (i.e., cigarette smoking cessation) should remain a high priority, a projection of lung cancer mortality through the end of the 20th century predicts that even with further reduction in smoking incidence, lung cancer in this decade will increase to a rate of 53.2 deaths per year per 100,000 population [2]. Clearly, new strategies for therapy are necessary.

The process of tumor growth and metastasis is complex and requires the highly orchestrated interactions of transformed neoplastic cells, tissue resident cells (i.e., fibroblasts, macrophages, and endothelial cells), and recruited cells (i.e., platelets, neutrophils, monocytes, and lymphocytes) from the circulation. One of the potential mechanisms that allows for maintenance of tumor growth is dysregulation of the balance of angiogenic and angiostatic factors. This dysregulation allows for the perpetuation of tumor growth and eventual metastasis. Based on a growing body of evidence, it is clear that a multitude of cytokines regulate growth. However, these cytokines may not express this full potential as individual polypeptides but collectively in a "cytokine network." To study all the pathophysiological aspects of cytokine networks during tumor growth and metastasis is a formidable task. Thus, in this review we will focus our studies on the expression and activity of members of the C-X-C chemokine family.

Abbreviations: NSCLC, nonsmall cell lung cancer; PF4, platelet factor 4; IFN, interferon; PBP, platelet basic protein; TP, total protein; SCID, severe combined immunodeficient; CTAP-III, connective tissue-activating protein-III; βTG, β-thromboglobulin; NAP-2, neutrophil-activating protein-2; IL-8, interleukin-8; GRO, growth-related oncogene; MIG, monokine induced by IFN-γ; IP-10, IFN-γ-inducible protein; ENA-78, epithelial neutrophil-activating protein-78; GCP-2, granulocyte chemotactic protein-2; bFGF, basic fibroblast growth factor.

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TUMORIGENESIS IS AN EXAMPLE OF EXAGGERATED WOUND REPAIR

For nonsmall cell lung cancer (NSCLC) growth to succeed within the host, a complex interplay must occur between transformed neoplastic cells and nontransformed resident and recruited immune and nonimmune cells (i.e., fibroblasts, endothelial cells, and subpopulations of leukocytes) [4]. Although carcinogenesis or neoplastic transformation is dependent on multiple genetic and epigenetic events [5], the salient feature of all solid tumor growth is the presence of neovascularization [6, 7]. In the absence of local capillary proliferation and delivery of oxygen and nutrients, neoplasms cannot grow beyond 1–2 mm^3 [6–8]. Folkman [7] first proposed in 1972 that tumors are angiogenesis dependent, with tumor growth correlating with a concomitant increase in vascular supply. In support of this contention is the finding that tumor cells contiguous with neovascularization have the highest [^{3}H]thymidine-labeling index, whereas, tumor cells further removed from capillaries have the lowest [^{3}H]thymidine-labeling index. Certain tumors have been found to produce factors that are directly angiogenic, whereas others may depend on neovascularization induced by products of resident cells or elicited leukocytes [7, 9–11]. These events are analogous to the formation of granulation tissue during the evolution of wound repair [4].

ANGIOGENESIS IS A PIVOTAL PROCESS OF TUMORIGENESIS

Angiogenesis is one of the most pervasive and essential biological events encountered in the mammalian organism [6, 12–14]. A number of physiological and pathological processes, such as embryonic development, the formation of inflammatory granulation tissue during wound healing, and the growth of malignant solid tumors, are strictly dependent on the recruitment of new capillaries. Normally, physiological angiogenesis occurs infrequently, yet can be rapidly induced in response to a number of diverse physiological stimuli. Among the most extensively studied of these angiogenesis-dependent physiological processes is normal wound repair [15]. An important feature of wound-associated angiogenesis is that it is locally transient and tightly controlled. The rate of capillary endothelial cell turnover is typically measured in months or years [16, 17]. However, when normally quiescent endothelial cells lining venules are stimulated, they will degrade their basement membrane and proximal extracellular matrix, migrate directionally, divide, and organize into new functioning capillaries invested by a new basal lamina all within a matter of days. This dramatic amplification of the microvasculature is nevertheless temporary, for as rapidly as they are formed they virtually disappear with similar swiftness, returning the tissue vasculature to homeostasis. The abrupt termination of angiogenesis that accompanies the resolution of the wound response suggests two possible mechanisms of control, neither of which are mutually exclusive. First, there is probably a marked reduction in angiogenic mediators. Second, a simultaneous increase occurs in the level of angiostatic factors that inhibit new vessel growth [8]. Although angiogenesis under conditions of normal wound repair appears to be under strict control, during neoplastic transformation neovascularization is exaggerated. It appears that tumors are continually renewing and altering their vascular supply [7]. Interestingly, a normal vascular mass of tissue is approximately 20%, whereas, during tumorigenesis tumor vascular mass may be 50% of the total tumor [7]. These findings are consistent with the observations that angiogenic activity is both a marker of preneoplastic-to-neoplastic transformation as well as an event that perpetuates tumorigenesis. In addition, the magnitude of tumor-derived angiogenesis has been directly correlated with metastasis of melanoma, prostate cancer, breast cancer, and NSCLC [7, 18–23]. Moreover, this would support the notion that tumor-associated angiogenesis is dysregulated in such a manner that a biological imbalance exists that favors either the overexpression of local angiogenic factors or the suppression of endogenous angiostatic factors [7, 18, 24]. Although most investigations studying angiogenesis have focused on the identification and mechanism of action of angiogenic factors, recent evidence suggests that angiostatic factors may play an equally important role in the control of neovascularization [8, 24–32].

ANGIOGENESIS IS REGULATED BY ANGIOSTATIC FACTORS

A role for inhibitors in the control of angiogenesis was first described with the observation that hyaline cartilage was particularly resistant to vascular invasion [24, 25]. These studies reported that a heat labile guanidium chloride extract prepared from cartilage contained an inhibitor of neovascularization. Later an identical extract from rodent neonatal and shark cartilage was able to effectively block neovascularization and growth of tumors in vivo [26, 27]. Similar inhibitors of angiogenesis have been reported for other cell and tissue extracts [26–30] and for a variety of natural and artificial agents including inhibitors of basement membrane biosynthesis [31–34], placental RNase inhibitor [35], lymphotoxin [36], interferons (IFNs) [37], prostaglandin synthetase inhibitors [38], heparin binding fragments of fibronectin [39], protamine [40], angiostatic steroids [41], several antineoplastic and anti-inflammatory agents [42, 43], and platelet factor 4 (PF4) [44]. Many of these compounds have several biological activities. For example, PF4 blocks immunosuppression and inhibits bone resorption. Although most inhibitors can act directly on the endothelial cell to block migration and/or mitogenesis in vitro, their effects in vivo may be considerably more complex, involving additional cells and their products.

AN IMBALANCE IN ANGIOGENIC AND ANGIOSTATIC FACTORS MAY CONTRIBUTE TO THE PATHOLOGY OF CHRONIC DISEASES

Several lines of evidence suggest that a biological imbalance in the production of angiogenic and angiostatic factors contributes to the pathogenesis of several angiogenesis-dependent disorders. For example, in rheumatoid arthritis the unrestrained proliferation of fibroblasts and neovascularization leads to the formation of prolonged and persistent granulation tissue whose degradative enzymes contribute to profound destruction of joint spaces [45–47]. In contrast, monocyte-derived

macrophage from patients with scleroderma fail to stimulate the expected angiogenic activity [48], suggesting that a defect in macrophage responsiveness may contribute to the attenuated neovascularization that is encountered in scleroderma. Psoriasis is a well-known angiogenesis-dependent disorder that is characterized by marked dermal neovascularization. We have recently reported that keratinocytes derived from psoriatic plaques are potentially angiogenic compared with normal keratinocytes. Interestingly, this aberrant phenotype is due, in part, to a combined defect in the overproduction of the angiogenic cytokine, IL-8, and a deficiency in the production of the angiogenesis inhibitor thrombospondin-1 [49–52]. Thus, in diseases such as rheumatoid arthritis, psoriasis, or tumorigenesis, neovascularization appears to be aberrantly up-regulated. Although angiogenic and angiostatic factors may vary among different physiological and pathological settings, the recognition of this dual mechanism for control of angiogenesis is necessary to gain a more thorough understanding of this complex process and its significance in promoting tumor growth.

C-X-C CHEMOKINE FAMILY OF CYTOKINES

The fidelity of inflammation and wound repair is dependent on cellular communication. Although this communication is often accomplished through direct cell-to-cell contact via specific cellular adhesion molecules, cells may signal one another through soluble mediators, such as cytokines. These polypeptide molecules often have pleiotropic effects on a number of biological functions including proliferation, differentiation, recognition, and cellular recruitment. Their actions are mediated through paracrine and autocrine signaling and under certain conditions they can behave as hormones. Recently, a new family of cytokines have been identified that appear to have proinflammatory and reparative activities [53–57]. These cytokines in their monomeric forms are all less than 10 kDa and are characteristically basic heparin binding proteins. This family displays four highly conserved cysteine amino acid residues, with the first two cysteines separated by one nonconserved amino acid residue. In general, these cytokines appear to have specific chemotactic activity for neutrophils. Because of their chemotactic properties and the presence of the C-X-C cysteine motif, these cytokines have been designated the C-X-C chemokine family. Interestingly, these chemokines are all clustered on human chromosome 4 and exhibit between 20% to 50% homology on the amino acid level [53–57]. Over the last decade, 12 different C-X-C chemokines have been identified and include PF4, NH_2-terminal truncated forms of platelet basic protein (PBP; connective tissue-activating protein-III [CTAP-III], β-thromboglobulin [βTG], and neutrophil-activating protein-2 [NAP-2]), interleukin-8 (IL-8), growth-related oncogene (GROα), GROβ, GROγ, IFN-γ-inducible protein (IP-10), monokine induced by IFN-γ (MIG), epithelial neutrophil-activating protein-78 (ENA-78), and granulocyte chemotactic protein-2 (GCP-2) [53–60]. The NH_2-terminal truncated forms of PBP are generated when PBP is released from platelet α-granules and undergoes proteolytic cleavage by monocyte-derived proteases. PF4, the first member of the C-X-C chemokine family to be described, was originally identified for its ability to bind to heparin, leading to the inactivation of heparin's anticoagulation function [61]. Both IP-10 and MIG are IFN-inducible C-X-C chemokines [58, 62]. Although IP-10 appears to be induced by all three interferons (IFN-α, IFN-β, and IFN-γ), MIG is unique in that it appears to be only expressed in the presence of IFN-γ [58]. Although IFN-γ induces the production of IP-10 and MIG, this cytokine has been found to attenuate the expression of both IL-8 and ENA-78 (63; and unpublished observations). These findings would suggest that members of the C-X-C chemokine family demonstrate disparate regulation in the presence of IFN-γ. GROα, GROβ, and GROγ and are closely related C-X-C chemokines, with GROα originally described for its melanoma growth stimulatory activity [64–66]. IL-8, ENA-78, and GCP-2 were all initially identified on the basis of their ability to induce neutrophil activation and chemotaxis [53–60]. IL-8 has been found to be produced by an array of cells including monocytes, alveolar macrophages, neutrophils, keratinocytes, mesangial cells, epithelial cells, hepatocytes, fibroblasts, and endothelial cells [53–57, 67–80]. Interestingly, IL-8 is expressed in neoplasms and produced by a number of transformed neoplastic cells [72, 75, 81–83]. Although numerous investigations have shown both in vivo and in vitro the importance of IL-8 in acute inflammation, as a chemotactic/activating factor for neutrophils, only recently has it become apparent that this C-X-C chemokine may be important in angiogenesis associated with tumorigenesis.

ROLE OF C-X-C CHEMOKINES IN ANGIOGENESIS

Our laboratory and others have found that IL-8 is a potent angiogenic factor [47, 84, 85]. Recombinant IL-8 mediates both endothelial cell chemotactic and proliferative activity in vitro and angiogenic activity in vivo. We found that IL-8 induced similar angiogenic activity as basic fibroblast growth factor (bFGF) [47]. Because monocytes/macrophages may represent a major source of angiogenic activity in wounds, other chronic diseases, and solid tumors [13], we extended our studies to determine whether IL-8 was a predominant angiogenic factor liberated by activated human monocytes or by synovial macrophages isolated from rheumatoid arthritis synovial tissues [47]. Conditioned medium from both populations of mononuclear phagocytes induced significant angiogenic activity that was attributable to IL-8 [47]. To further demonstrate that the angiogenic effect was attributable to IL-8, we used an IL-8 antisense oligonucleotide strategy to inhibit the production of IL-8 at the pretranslational level [47]. The conditioned medium from monocytes treated in the presence of IL-8 antisense inhibited endothelial chemotactic activity by 84% compared with the IL-8 sense oligonucleotide-treated monocytes. Similar results were found in the in vivo corneal micropocket model of angiogenesis. These findings indicated that IL-8, at concentrations of approximately 1 nM, functioned as a mediator of angiogenesis. This amount of IL-8 compares with amounts reported for the induction of corneal angiogenic activity by tumor necrosis factor-α, aFGF, bFGF, angiogenin, angiotropin, and endothelial cell growth factor [47].

Another member of the C-X-C chemokine family, PF4, has been shown to have angiostatic properties in nanomolar to micromolar concentrations [44] and to attenuate the growth of tumors in vivo [86]. Interestingly, these studies had initially demonstrated that the angiostatic ac-

tivity of PF4 was due to its heparin binding domain (within the COOH-terminal of the molecule) [44, 86]. However, recent studies have now shown that a PF4 mutant that lacks the heparin binding domain, and functional heparin binding is equipotent in vivo to native PF4 for the attenuation of tumor growth [87]. The above findings would suggest that members of the C-X-C chemokine family function as either angiogenic or angiostatic factors in regulating neovascularization, and the biological balance in the magnitude of expression of these angiogenic and angiostatic C-X-C chemokines within a tumor could dictate overall tumor-derived angiogenic activity. Although it remains unclear whether the COOH-terminal of these chemokines dictates their biological role in angiogenesis, the differences in C-X-C chemokine function could also be explained by other structural domains. Recently, both Hébert et al. [88] and Clark-Lewis et al. [89] have demonstrated a salient amino acid sequence in the primary structure of the C-X-C chemokine family that appears, in part, to account for the ability of these chemokines to function in neutrophil chemotaxis and activation. They demonstrated that the three amino acid residues that immediately preceded the first cysteine amino acid are critically important in the binding and activation of neutrophils. These amino acids are Glu-Leu-Arg, the ELR motif, which is absent in certain members of the C-X-C chemokine family (PF4, IP-10, and MIG) that display markedly reduced potency in mediating neutrophil chemotaxis. Interestingly, when the ELR motif was introduced into PF4, this chemokine gained 1000-fold potency in mediating neutrophil chemotaxis [89]. Thus, these structural differences, in part, may explain the disparity of angiogenic activity of the C-X-C chemokine family and support the hypothesis that a biological imbalance in the expression of angiogenic and angiostatic C-X-C chemokines may lead to the perpetuation of neovascularization during tumor growth and metastasis.

ROLE OF IL-8 AS AN ANGIOGENIC FACTOR IN NSCLC

Previous studies have demonstrated that human tumors and neoplastic cell lines may directly elaborate IL-8 [72, 75, 81–83, 90–92]. Because angiogenesis is critical to tumorigenesis and metastasis, we extended our initial discovery of IL-8 as an angiogenic factor to assess whether this C-X-C chemokine was present in NSCLC and whether it contributed to overall tumor-derived angiogenic activity [93]. We found significantly elevated IL-8 levels in natural human NSCLC, by using a specific ELISA, that were fourfold greater than normal lung tissue [93]. Results from IL-8 immunohistochemistry of tumor sections confirmed a heterogeneous pattern of tumor cell production of IL-8 from both adenocarcinomas and squamous cell carcinomas of the lung [93]. The heterogeneity of the tumor cell expression was of interest, especially because we had previously shown that only 36% to 38% of adenocarcinoma cells (A549 cell line) expressed IL-8 [75]. The findings of heterogeneous expression of IL-8 by tumor cells suggests that specific subclones of neoplastic cells may exist and function as the primary cellular source of tumor-derived IL-8. Our results also revealed that nontransformed stromal cells within the host desmoplastic response to the tumor were also serving as significant cellular sources for IL-8, especially in response to the squamous cell carcinoma [93]. Importantly, these specific findings may reflect the different clinical behavior of squamous cell and adenocarcinomas. The more aggressive course of adenocarcinomas could be related to their capacity to generate a sufficient angiogenic signal [IL-8], independent from the surrounding host-responding immune and nonimmune cells. The observation of a minimal inflammatory cell infiltrate in the tumor specimens, despite the presence of IL-8, was unexpected and supported the contention that IL-8 may have other biological functions in the context of NSCLC.

Because it was apparent that NSCLC was associated with significantly elevated levels of IL-8, we next determined whether IL-8 contributed to overall tumor-derived angiogenic activity. By using neutralizing antibodies to IL-8, we found that IL-8 accounted for 42% to 80% of the angiogenic activity for each of the tumor specimens, as determined by bioassays of angiogenesis [93]. The above data suggested that a significant portion of tumor-derived angiogenic activity was mediated directly by IL-8. Although IL-8-dependent angiogenic activity represented a significant proportion of overall NSCLC-derived angiogenesis, we wanted to compare the relative contribution of IL-8 to 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, respectively, for adenocarcinoma, squamous cell carcinoma, and A549 (adenocarcinoma) samples. In contrast, anti-bFGF antibodies had no significant effect on the endothelial cell chemotaxis in response to samples of A549 cells/tissue or squamous cell carcinoma tissue; however, neutralizing anti-bFGF antibodies reduced the endothelial cell chemotactic activity from adenocarcinoma tissue by 35% of the standard bioactivity. Interestingly, the neutralization of transforming growth factor-α had no significant effect on the chemotaxis in response to adenocarcinoma or to the A549 cell/tissue; however, these antibodies resulted in a significant reduction (45%) in the endothelial cell chemotactic response to squamous cell carcinoma tissue. Although bFGF and transforming growth factor-α have been previously described as potential angiogenic factors involved in tumor angiogenesis [4, 7, 92, 94, 95], these studies were the first to demonstrate that a primary angiogenic signal for NSCLC neovascularization was directly mediated by tumor-associated IL-8.

ROLE OF C-X-C CHEMOKINES IN MEDIATING ANGIOGENESIS IN THE CONTEXT OF TUMORIGENESIS: OUR HYPOTHETICAL MODEL

The experiments designed in our laboratories have focused on the role of angiogenic and angiostatic C-X-C chemokines and whether a biological imbalance in their expression favors tumor-derived angiogenic activity. We hypothesize that angiogenesis associated with NSCLC tumor growth is dependent on members of the C-X-C chemokine family acting as either angiogenic or angiostatic factors (**Fig. 1**). This paradigm predicts that a shift in the balance of expression of these C-X-C chemokines dictates whether the neoplasm grows and develops metastatic potential or regresses. The net angiogenic activity during the progression of tumorigenesis is mediated by the biological imbalance that favors the expression of

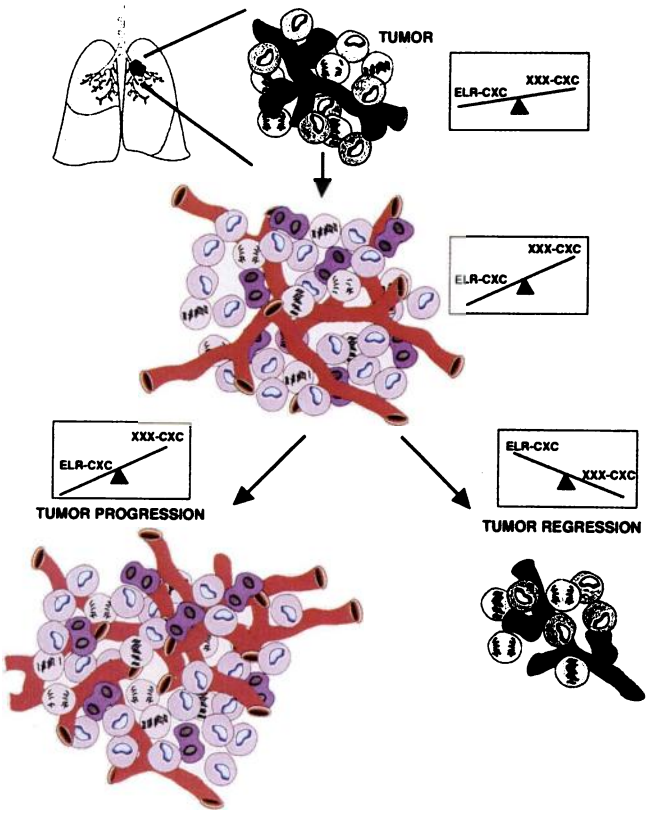

Fig. 1. The role of C-X-C chemokines in mediating angiogenesis in the context of tumor genesis: our hypothetical model. ELR, chemokines containing the ELR motif; XXX, chemokines that lack the ELR motif.

angiogenic C-X-C chemokines (ELR-C-X-C) compared with the angiostatic C-X-C chemokines (XXX-C-X-C). However, the magnitude of expression and the biological imbalance will favor the production of angiogenic C-X-C chemokines (ELR-C-X-C). This effect results in tumor growth, invasion, and metastasis beyond the confines of its primary site of origin. In contrast, the generation of IP-10 and MIG will have a negative effect on tumorigenesis through a reduction in tumor-derived angiogenic activity. These C-X-C chemokines are primarily induced by IFN-γ, which is known to be attenuated by IL-10. The production of IL-10 by both neoplastic cells and surrounding nontransformed immune and nonimmune cells will have a significant, yet indirect, impact on the generation of angiostatic (XXX-C-X-C) chemokines (IP-10 and MIG). Under these circumstances, IL-10 expression in the context of tumorigenesis can be viewed as an indirect promoter of angiogenesis via the down-regulation of IFN-γ. The experimental model of human NSCLC/SCID mouse chimera has provided the opportunity to test whether either attenuation of angiogenic (ELR-C-X-C) chemokines or accentuation of angiostatic (XXX-C-X-C) chemokines promotes tumor regression.

PRELIMINARY STUDIES

The major aims of our experiments were to study the role of C-X-C chemokines as they relate mechanistically to the pathophysiological consequences of tumorigenesis. The following preliminary data demonstrates that specific members of the C-X-C chemokine family of chemotactic cytokines are either angiogenic or angiostatic mediators of neovascularization. In addition, these data support the notion that these C-X-C chemokines are likely candidates to target novel future specific therapies.

C-X-C CHEMOKINES (ELR MOTIF) INDUCE AND IP-10 AND PF4 (NON-ELR MOTIF) ATTENUATE ANGIOGENESIS

We postulated that the members of the C-X-C chemokine family may exert disparate effects in mediating angiogenesis for primarily four reasons. First, members of the C-X-C chemokine family that display binding and activation of neutrophils share the same ELR motif homology that immediately precedes the first cysteine amino acid residue, whereas, PF4, IP-10, and MIG lack this motif [88, 89]. Second, IL-8 (contains ELR motif) is angiogenic and PF4 (lacks the ELR motif) is angiostatic. Third, the IFNs (IFN-α, IFN-β, and IFN-γ) are all known inhibitors of wound repair, especially angiogenesis and reepithelialization [37, 92, 95–110], however, these cytokines up-regulate IP-10 and MIG from a number of cells, including keratinocytes, fibroblasts, endothelial cells, and mononuclear phagocytes [58, 62]. We hypothesize that IFNs may be exerting their negative influence in wound repair by up-regulating the production of IP-10 and MIG, which may act in an autocrine and paracrine manner to suppress angiogenesis. Finally, we and others have found that IFN-α, IFN-β, and IFN-γ are potent inhibitors of both monocyte-derived IL-8 and ENA-78 (63; and unpublished observations), supporting the notion that IFN-α, IFN-β, and IFN-γ may shift the biological balance of C-X-C chemokines toward a preponderance of angiostatic C-X-C chemokines. To test this postulate, we performed endothelial cell chemotaxis in the presence or absence of C-X-C chemokines that contain the ELR motif (IL-8, ENA-78, GCP-2, GROα, GROβ, GROγ, PBP, CTAP-III, and NAP-2) and C-X-C chemokines lacking the ELR motif (IP-10 and PF4). In a similar fashion to IL-8, all of the C-X-C chemokines that contained the ELR motif demonstrated significant endothelial cell chemotactic activity, whereas the endothelial cell chemotactic activity induced by either IP-10 or PF4 were similar to control (background). In addition, ENA-78, GCP-2, GROα, GROβ, GROγ, and NAP-2 in a similar concentration to IL-8 demonstrated significant corneal neovascularization without any evidence for inflammatory cellular infiltration.

To delineate whether IP-10 (C-X-C chemokine lacking the ELR motif) could modulate C-X-C chemokine (ELR motif present)-induced endothelial cell chemotactic activity, IL-8, GROα, CTAP-III, and NAP-2 were assessed for endothelial cell chemotaxis in the presence of IP-10. The presence of IP-10 significantly attenuated endothelial cell chemotactic activity in response to C-X-C chemokines containing the ELR motif. In addition, PF4 in a similar fashion to IP-10 also attenuated C-X-C chemokine (ELR motif present)-induced endothelial cell chemotactic activity. Furthermore, by using the corneal micropocket model of neovascularization, IP-10 in equivalent molar concentrations was found to attenuate IL-8-, ENA-78-, GCP-2-, and GROα-induced angiogenesis. These findings support the premise that members of the C-X-C chemokine family may be acting as either angiogenic or

TABLE 1. C-X-C Chemokines Display Disparate Angiogenic Activity

Angiogenic C-X-C chemokines
IL-8
ENA-78
GCP-2
GROα
GROβ
GROγ
CTAP-III
βTG
NAP-2
Angiostatic C-X-C chemokines
PF4
IP-10
MIG

angiostatic factors (**Table 1**). The net biological balance of these C-X-C chemokines, in the context of tumorigenesis, may play an important role in modulating NSCLC neovascularization.

PRESENCE OF C-X-C CHEMOKINES IN NSCLC

The evidence that C-X-C chemokines could function as either angiogenic or angiostatic factors, depending on the presence of the ELR motif, lead to our investigation as to whether C-X-C chemokines were present in natural human NSCLC and whether they contributed to overall NSCLC tumor-derived angiogenic activity. IL-8, ENA-78, and GROα were found in a 4-, 3-, and 2.5-fold excess, respectively, in tumor tissue compared with normal lung tissue normalized to total protein (TP). Normal lung tissue contained 2.6 ± 0.7, 15 ± 6, and 6 ± 2.5 ng/mg TP of IL-8, ENA-78, and GROα compared with 11.9 ± 3.8, 43.6 ± 10, and 11.5 ± 2.5 ng/mg TP for tumor specimens, respectively. There were similar elevations of IL-8 and ENA-78 from both adenocarcinomas and squamous cell carcinomas, whereas GROα was found to be especially elevated in squamous cell (17.6 ± 7.8 ng/mg TP) carcinomas. In contrast, PF4 levels were found in the carcinoma tissue homogenates to be equivalent to normal lung tissue (592.4 ± 127.8 ng/mg TP and 579.1 ± 195.3 ng/mg TP for tumors and normal lung tissue, respectively). Although IP-10 levels trended toward being greater in tumors than in normal lung tissue homogenates, IP-10 was significantly lower in adenocarcinomas (0.9 ± 0.5 ng/mg TP) than in normal lung tissue (3.6 ± 2 ng/mg TP). Importantly, these specific findings may be reflected in the different clinical behaviors of squamous cell carcinoma and adenocarcinoma NSCLCs. The more aggressive course of adenocarcinomas could be related to their capacity to generate a greater angiogenic compared with angiostatic C-X-C chemokine signal. To further substantiate that IP-10 may be acting as an endogenous angiostatic C-X-C chemokine to balance the effect of angiogenic factors in the context of NSCLC, we assessed squamous cell carcinoma tissue homogenates for angiogenesis in the presence of neutralizing IP-10 or control antibodies. By using either endothelial cell chemotaxis or a corneal micropocket model of neovascularization, we found that neutralizing IP-10 antibodies (without evidence of lipopolysaccharide contamination) significantly augmented tumor-derived angiogenic activity by twofold. These findings support the presence of an imbalance in ELR-C-X-C compared with XXX-C-X-C chemokines, with the balance favoring a greater presence of angiogenic C-X-C chemokines in NSCLC tumors.

HUMAN NSCLC/SEVERE COMBINED IMMUNODEFICIENT (SCID) MOUSE CHIMERA AS A MODEL FOR HUMAN TUMORIGENESIS

Although neoplastic transformation is dependent on multiple genetic and epigenetic events [5], the success of tumorigenesis is dependent on the complex biological interplay between the neoplastic cells and the resident and recruited host-responding cells. For example, in the absence of local neovascularization these neoplasms could not grow beyond 1-2 mm^3 [6-8]. Thus, to effectively study the complex biology of human solid tumors, the use of human tumor xenografts in immunodeficient mice have provided significant insight into the biology of tumor growth and metastasis [111-120]. Our preliminary data supports the notion that specific members of the C-X-C chemokine family are important cytokines involved in orchestrating a significant portion of the angiogenic response during tumorigenesis and that other members may be important in attenuating this response. To characterize the qualitative and quantitative presence of these angiogenic and angiostatic C-X-C chemokines, as well as determine their net contribution to human NSCLC tumorigenesis in vivo, we have transplanted either intact natural human NSCLC or NSCLC cell lines (A549, adenocarcinoma and Calu-1, squamous cell carcinoma) into SCID mice to create a human NSCLC/SCID mouse chimera. The mice were evaluated before xenograft to have a murine serum Ig concentration of <1 μg/ml by ELISA. Freshly isolated intact NSCLC (adenocarcinoma) were cut into 1 mm^3 and placed subcutaneously into the bilateral flanks of SCID mice and allowed to grow for 4 weeks. At this time point the tumors measured 4-9 mm in diameter and the mice were killed. Immunohistochemistry for IL-8 and ENA-78 demonstrated the immunolocalization of these C-X-C chemokines within the viable NSCLC cells, stromal cells, and mononuclear cells, whereas the control antibodies failed to show nonspecific staining. These findings suggested that natural human NSCLC remained viable in SCID mice and that they continue to express both IL-8 and ENA-78 that may have allowed them to generate angiogenic activity and survive in the SCID mouse host.

Because our central hypothesis is that overall tumor-derived angiogenesis is dependent on an imbalance in the production of members of the C-X-C chemokine family that act as angiogenic rather than angiostatic factors, we next assessed in vitro whether human NSCLC cell lines (A549 and Calu-1) produced disparate levels of angiogenic (IL-8, ENA-78, and GROα) and angiostatic (PF4 and IP-10) C-X-C chemokines. Both A549 and Calu-1 human NSCLC cell lines were found to constitutively produce high levels of angiogenic C-X-C chemokines. Interestingly, the A549 cells were found to constitutively produce greater levels of angiogenic C-X-C chemokines, and when grown in SCID for 4 weeks, were found to induce tumors twofold greater in size than the Calu-1 NSCLC cell line. These findings corroborate the previous findings that A549 cells display greater tumorgenicity and spontaneous metastasis than Calu-1 cells in vivo [119-121]. Although

these in vitro studies were important to establish whether an imbalance in the production of angiogenic compared with angiostatic C-X-C chemokines existed in NSCLC cell lines, we next assessed whether an imbalance in their production occurred in vivo during tumorigenesis of A549 NSCLC cells in a SCID mouse host.

SCID mice received 10^6 A549 cells suspended in 100 μl of phosphate-buffered saline to each flank. The mice were killed in a time-dependent manner at 2, 3, 4, 5, 6, and 7 weeks postxenograft and tumors were analyzed for tumor growth, and tumor-derived and plasma levels of C-X-C chemokines standardized to either TP or volume (biopsy). Immunolocalization of IL-8 from A549 tumors at week 7 demonstrated a heterogeneous expression pattern with 35 ± 6% of the cells expressing IL-8 protein by image analysis, whereas IL-8 expression from spontaneous A549 tumor metastasis to the lungs of SCID mice at 7–8 weeks demonstrated a more homogenous pattern of immunolocalization of IL-8, with more than 80% of the cells expressing IL-8 protein by image analysis. Moreover, when A549 tumors in SCID mice and plasma from these mice were analyzed for the presence of C-X-C chemokines, we found a significant correlation of the temporal expression of angiogenic compared with angiostatic C-X-C chemokines during tumorigenesis. These studies substantiated that the production of angiogenic C-X-C chemokines paralleled tumor growth and supported our contention that an imbalance in angiogenic and angiostatic C-X-C chemokines exist during tumorigenesis. However, these studies did not demonstrate whether these angiogenic C-X-C chemokines directly contributed to an imbalance favoring net angiogenesis leading to tumor growth or tumor survival. To assess whether IL-8 directly contributed to tumor growth and survival, human NSCLC (A549) tumorigenesis was allowed to occur in SCID for a period of 4 weeks before the initiation of in vivo passive immunization with neutralizing antibodies to IL-8 (every other day during weeks 5, 6, and 7 of tumor growth). We found the tumors (at 8 weeks) to demonstrate markedly more central necrosis in animals that had received neutralizing antibodies to IL-8 compared with animals receiving control antibodies. In addition, morphological analysis, thresholded to NSCLC cell nuclear size, showed a significant reduction in cellularity of the tumors that were exposed to neutralizing IL-8 antibodies. The above findings provide evidence that supports our contention that the ELR motif containing C-X-C chemokines are important angiogenic molecules during tumorigenesis, and that an imbalance in their expression compared with angiostatic C-X-C chemokines, dictates their role in mediating net tumor-derived angiogenesis during tumorigenesis. Furthermore, the use of the human NSCLC/SCID mouse model has allowed us to validate our central hypothesis and examine the biology of C-X-C chemokines as they relate to angiogenesis in vivo. This model of human tumorigenesis has allowed the unique opportunity to examine the temporal magnitude, cellular sources, and contribution of the angiogenic and angiostatic C-X-C chemokines to the evolution of tumorigenesis and spontaneous metastasis.

CYTOKINE NETWORKS THAT MAY PROMOTE ANGIOGENIC C-X-C CHEMOKINES

The above preliminary data coupled with what is known regarding the role of IFNs in wound repair, suggest that the magnitude of IFN expression would be a pivotal event in locally regulating both angiogenic (through negative feedback) and angiostatic (through positive feedback) C-X-C chemokine production. IFNs are pleiotropic cytokines that exert a broad range of immunomodulatory and inflammatory effects, however, they share a salient feature of cellular growth inhibition. IFN-α and IFN-β are produced primarily by mononuclear phagocytes and fibroblasts, respectively, whereas IFN-γ is produced by $CD4^+$ and $CD8^+$ T cells and natural killer cells [109, 110]. These IFNs are all known inhibitors of wound repair, specifically they appear to have a significant inhibitory influence on endothelial cell biology [37, 90, 95, 102–110]. This supports the notion that IFNs can modulate suppression of neovascularization through both direct and indirect pathways. Although IFN-α, IFN-β, and IFN-γ can inhibit the cellular expression of angiogenic C-X-C chemokines, these IFNs are important for the induction of angiostatic C-X-C chemokines (IP-10 and MIG). The net effect of IFN stimulation would be to shift the biological balance in favor of angiostatic C-X-C chemokines. This potential mechanism could be operative during the later stages of normal wound repair, where new capillary blood vessel formation is rapid, under strict control, and undergoes marked regression to a physiological steady-state level. In contrast, angiogenesis during neoplastic transformation is exaggerated [7, 8]. This perpetual neovascularization during tumorigenesis suggests two possible pathological mechanisms, neither of which are mutually exclusive: (1) tumorigenesis is associated with an increase in the synthesis and/or elaboration of angiogenic mediators and (2) tumorigenesis is associated with reduced levels of substances that inhibit neovascularization. Because our preliminary data supported the former, we also postulated that these neoplasms may be associated with equivalent or reduced levels of IFN-γ compared with normal lung tissue, which would support the latter. To test this hypothesis, we analyzed human tissue homogenates of both normal and NSCLC for the presence of IFN-γ by using an IFN-γ specific ELISA. IFN-γ concentrations from tumor samples were found to be similar to normal lung tissue. These findings corroborated the study by Vitolo et al. [122] who found that cytokine expression of mRNA (TH-1 profile) from tumor-infiltrating lymphocytes was unexpectedly reduced within solid tumors compared with other chronic inflammatory disorders. The above findings may reflect the reduced immunogenicity of tumor-associated antigens, however, an alternative explanation may be the ability of the tumor or host-responding cells to produce immunosuppressive factors that may directly impact on the local production of IFN-γ.

IL-10 is a recently characterized cytokine that demonstrates varied immunosuppressive bioactivity. Since its initial isolation by Mosmann et al. [123–126] in 1988, investigations have elucidated many of the immunological properties of this cytokine. Originally identified as a product of $CD4^+$ T cells, IL-10 is also produced by monocytes, macrophages, B cells, certain populations of $CD8^+$ T cells, and Epstein-Barr virus-transformed lymphoblastoid cells lines [123, 124, 126–130]. Recent work has demonstrated that epidermal cells may also elaborate IL-10 [131]. Functional studies reveal that IL-10 has profound effects on monocytes, resulting in alterations in cell morphology and cytotoxicity, down-regulation of the expression of major histocompatibility complex class II antigens, and

inhibition of proinflammatory cytokine production [132–137]. Furthermore, IL-10 also exerts direct effects on the growth and function of T cells, B cells, and mast cells [123, 124]. These specific actions result in the capacity for IL-10 to attenuate a wide range of effector immune responses, including T cell cytokine (i.e., IFN-γ) production and antigen-specific proliferation, B cell Ig synthesis, and the elaboration of tumor necrosis factor-α and IFN-γ by natural killer cells [123, 124]. IL-10 may play an important role in homeostasis under normal circumstances; however, IL-10, in the context of tumorigenesis, may be a major immunosuppressive factor that attenuates the local generation of IFN-γ. To test this premise, we investigated the capacity of NSCLC to produce IL-10 [138]. We found increased levels of antigenic IL-10 in tissue homogenates of NSCLC (13.7 ± 2.8 ng/mg TP) compared with normal lung tissue (5.8 ± 0.8 ng/mg TP). No significant difference in IL-10 levels were seen between the squamous cell carcinoma (15.4 ± 3.7 ng/mg TP) and adenocarcinoma (12.3 ± 4.2 ng/mg TP). To determine the cellular source of IL-10 in the NSCLCs, immunohistochemical staining of NSCLC showed primary localization of antigenic IL-10 to individual NSCLC cells [138]. In addition, immunolocalization using HAM56, (Enzo Diagnostics, Inc., Farmingdale, NY), a murine monoclonal antibody against human mononuclear cells, confirmed that tumor cells, rather than immune cells, were the primary cellular source of antigenic IL-10 [138]. Because IL-10 had previously been demonstrated to be produced by epidermal cells [131], we analyzed the conditioned medium of several unstimulated human NSCLC cell lines (A549, A427, and Calu-6) for the constitutive production of IL-10. These cells produced 6.3 ± 1 ng/ml, 1.9 ± 0.9 ng/ml, and 7.6 ± 1.1 ng/ml of IL-10 after 24 h of culture, respectively [138]. These findings demonstrate that NSCLC cell lines can elaborate IL-10. Thus, IL-10 may play an important role in impairing immune cell effector function and enable the NSCLC to evade host immune defenses; however, IL-10 suppression of IFN-γ may play an equally important role in perpetuating tumor-associated neovascularization.

Our data demonstrates and supports the potential presence of an imbalance in the expression of angiogenic and angiostatic C-X-C chemokines during tumorigenesis that favors the tumor-derived angiogenesis. In addition, the presence of augmented levels of IL-10 in NSCLC may favor the notion that IL-10 may promote angiogenesis via a direct role in attenuating IFN-γ and therefore, indirectly inhibiting angiostatic C-X-C chemokines during tumorigenesis. The further elucidation of the biology of these cytokines will provide new insight into the specific aspects of C-X-C chemokine biology responsible for NSCLC angiogenesis and subsequent tumorigenesis and metastasis.

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