

## Inhibition of Interleukin 8 Attenuates Angiogenesis in Bronchogenic Carcinoma

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### Summary

We investigated the role of interleukin 8 (IL-8) in mediating angiogenesis in human bronchogenic carcinoma. Increased quantities of IL-8 were detected in tumor tissue as compared with normal lung tissue. Immunohistochemical staining of tumors revealed primary localization of IL-8 to individual tumor cells and demonstrated the capacity of tumor to elaborate IL-8. Functional studies that used tissue homogenates of tumors demonstrated the induction of both in vitro endothelial cell chemotaxis and in vivo corneal neovascularization. It is important to note that the addition of neutralizing antisera to IL-8 to these assays resulted in the marked and specific attenuation of these responses. Our observations definitively establish IL-8 as a primary mediator of angiogenesis in bronchogenic carcinoma and offer a potential target for immunotherapies against solid malignancies.

The leading cause of malignancy-related mortality in the United States is bronchogenic carcinoma (1). While the incidence of many 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 are attributable to bronchogenic carcinoma in the U.S. annually (1). Despite attempts to advance early diagnosis and employ combination therapies, the clinical response of this tumor yields an overall 5-yr survival rate for lung cancer patients of <15%. A new approach to therapy is clearly needed.

Tumor vascularization is crucial for the pathogenesis of all solid malignancies. In the absence of local capillary proliferation, tumors may not grow beyond 2–3 mm in diameter (2). Folkman first proposed in 1972 that tumors are angiogenesis-dependent, with tumor growth requiring concomitant increases in vascular supply. Previous studies have attempted to investigate the complexities of tumor neovascularization (3). Certain tumors have been found to produce factors that are directly angiogenic, whereas others may depend upon vascularization induced by products of responding inflammatory cells (4–6). IL-8 is a cytokine of the C-X-C supergene family that has been classified primarily by pro-inflammatory and leukocyte chemotactic properties (7–9). A variety of cell populations, including neutrophils (10), mononuclear (11, 12), stromal (13), endothelial (14), and ep-

ithelial cells (15), and hepatocytes (16) may serve as sources for IL-8. The recent characterization of potent angiogenic properties has renewed interest in the role of IL-8 in a variety of settings (17, 18). In the present study we demonstrate IL-8 is a primary mediator of angiogenesis in human bronchogenic carcinoma.

### Materials and Methods

*Antigenic Determination of IL-8 Content of Normal Lung and Bronchogenic Tumor Tissue.* Tissue specimens were obtained from consenting individuals undergoing thoractomy for suspected primary bronchogenic carcinoma in accordance with the University of Michigan I.R.B. approval. Samples of tumor, and normal lung distal to tumor, were homogenized in PBS upon recovery from the operating room. Specimens were then filtered through 0.45-micron Sterile Acrodiscs (Gelman Sciences Inc., Ann Arbor, MI) and frozen at  $-70^{\circ}\text{C}$  until thawed and then assayed. A specific ELISA for IL-8 was employed to determine IL-8 content of tissue samples as previously described (19). Samples were run in parallel for total protein content (Pierce Chemical Co., Rockford, IL). Determinations were expressed as nanograms of IL-8 per milligrams total protein. Results were tabulated based on final histological diagnosis as determined by University Hospital pathologists.

*Immunohistochemical Localization of Antigenic IL-8.* Fresh tissue specimens obtained at time of thoractomy were fixed in 4% paraformaldehyde for 24 h before transferring to 70% ethanol. Paraffin-embedded tissue sections were dewaxed with xylene and rehydrated

through graded concentrations of ethanol. Samples were then stained for IL-8 using a modification of our previously described technique (20). Briefly, nonspecific binding sites were blocked with normal goat serum (BioGenex Laboratories, San Ramon, CA) before washing and applying a 1:1,000 concentration of either control (rabbit) or rabbit anti-human IL-8 serum. Slides were then rinsed and overlaid with secondary biotinylated goat anti-rabbit IgG (1:35) and then incubated. After washing twice with Tris-buffered saline, slides were overlaid with a 1:35 dilution of alkaline phosphatase conjugated to streptavidin (BioGenex Laboratories), and incubated. Fast Red (BioGenex Laboratories) reagent was used for chromogenic localization of IL-8 antigen. After optimal color development, sections were immersed in sterile water, counterstained with Mayer's hematoxylin, and coverslipped using an aqueous mounting solution.

**Endothelial Cell Chemotaxis Responses to IL-8 Standard and Tissue Specimens.** Endothelial cell chemotaxis was performed in 48-well, blind well chemotaxis chambers (Nucleopore Corp., Pleasanton, CA) as previously described (21). Nucleopore Chemotaxis membranes (5 micron pore size; Nucleopore Corp.) were prepared by soaking them sequentially in 3% acetic acid overnight and for 2 h in 0.1 mg/ml gelatin. Membranes were rinsed in sterile water, dried under sterile air, and stored at room temperature for up to 1 mo. Bovine adrenal gland capillary endothelial cells (BCE),<sup>1</sup> maintained in gelatin-coated flasks in DME with 10% FCS were used as the target cells. 24 h before use BCE were starved in DME with 0.1% BSA. 25  $\mu$ l of cells, suspended at a concentration of 10<sup>6</sup> per ml in DME with 0.1% BSA were dispensed into each of the bottom wells. A chemotaxis membrane was positioned atop the bottom wells, chambers were sealed, inverted, and incubated for 2 h to allow cells to adhere to the membrane. Chambers were then re-inverted, 50  $\mu$ l test media was dispensed into the top wells and re-incubated for an additional 2 h. Membranes were then fixed and stained with a Diff-Quick staining kit (American Scientific Products, McGraw Park, IL) to enumerate membrane-bound cells, and cells that had migrated through the membrane to the opposite surface were counted. Four replicates, 10 fields/replicate, were tested for each sample, and experiments were repeated at least three times. Results were expressed as the total number of endothelial cells that migrated across the filter in 10 high power (400 $\times$ ) fields. IL-8 neutralization studies used our previously well-characterized polyclonal rabbit anti-human IL-8 antibody (13, 18, 20). Additional neutralization studies employed the commercially available neutralizing goat anti-human TGF- $\alpha$  antibody and rabbit anti-basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN).

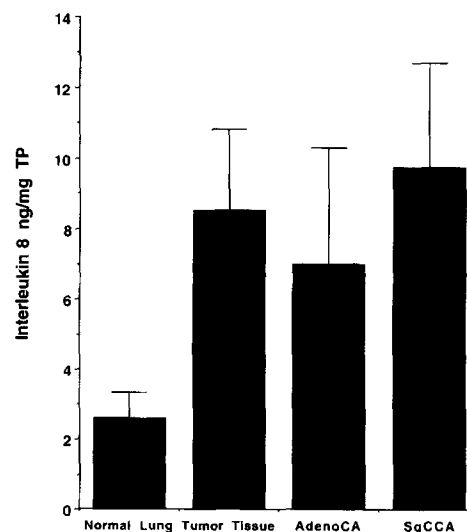
**Corneal Micropocket Model of Angiogenesis.** In vitro angiogenic activity was assayed in the avascular cornea of F344 female rats eyes, as previously described (22, 23). Briefly, 5 mg total protein of each specimen was combined with an equal volume of sterile Hydron casting solution, and 5-ml aliquots were pipetted onto the surface of 1-mm Teflon rods glued to the surface of a glass petri dish. Pellets were air-dried in a laminar flow hood (1 h) and refrigerated overnight. Before implantation pellets were rehydrated with a drop of lactated Ringer's solution. Animals were anesthetized with metofane and injected with sodium pentobarbital intraperitoneally. A retrobulbar injection of 0.1 ml of 2% lidocaine was made before intracorneal implantation of the Hydron pellet into a surgically created intracorneal pocket  $\sim$ 1.5 mm from the limbus. The animals were examined daily with a stereomicroscope. 7 d after implantation, animals were re-anesthetized and perfused sequentially with

lactated Ringer's solution followed by colloidal carbon. Corneas were harvested, flattened, and photographed (33 $\times$ ). Positive neovascularization responses were recorded only if sustained directional ingrowth of capillary sprouts and hairpin loops towards the implant were observed. Negative responses were recorded when either no growth was observed or when only an occasional sprout or hairpin loop displaying no evidence of sustained growth was detected. Animals were handled in accordance with the University of Michigan U.L.A.M. protocols.

## Results and Discussion

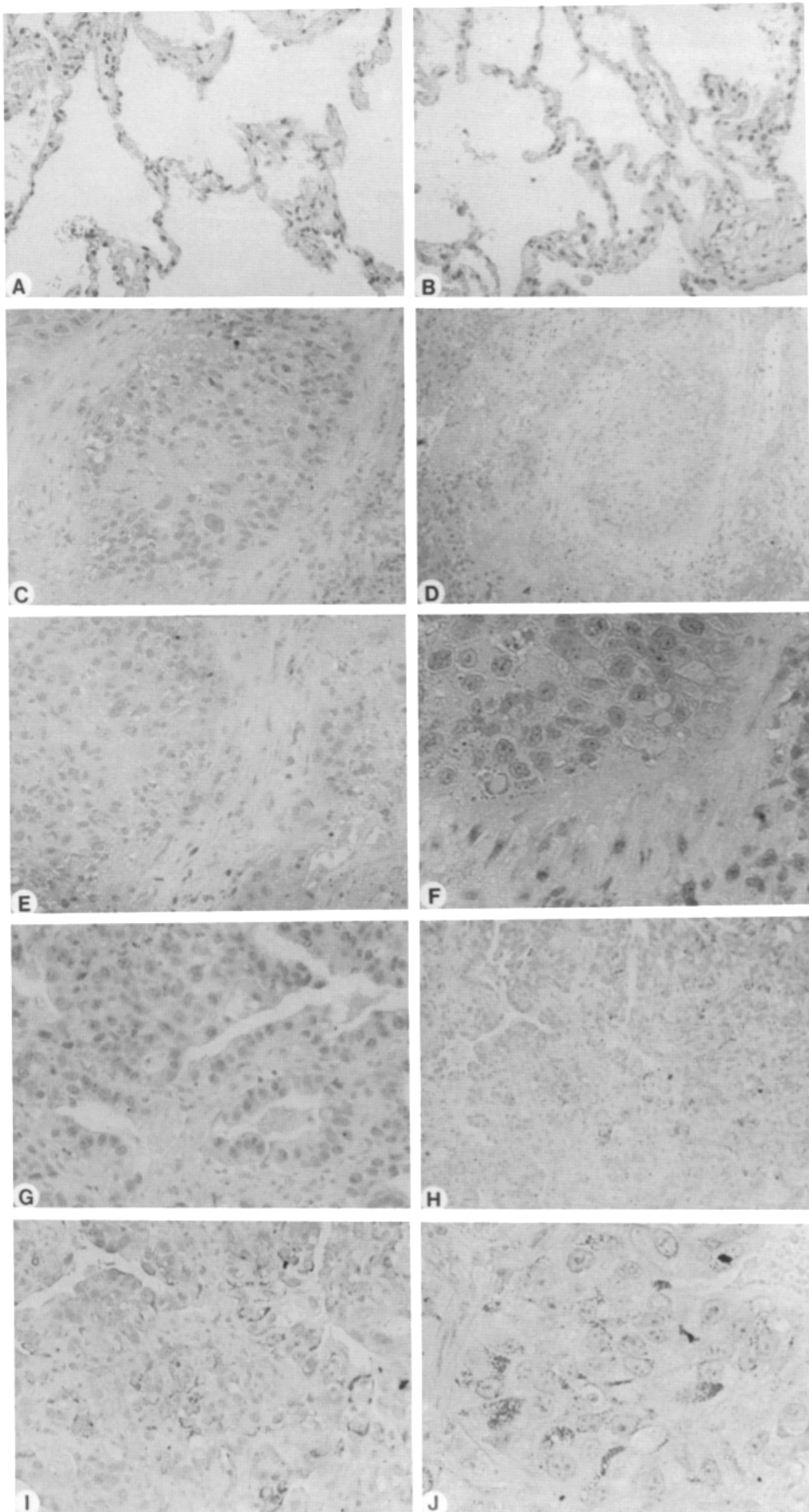
**IL-8 Is Increased in Tumor Tissue.** We began our evaluation using an ELISA to detect IL-8 in human tissue homogenates of both normal lung and nonsmall cell bronchogenic carcinomas. As shown in Fig. 1, antigenic IL-8 was observed in a fourfold excess in tumor tissue as compared with normal lung tissue (expressed as nanograms per milligrams of total protein [TP]). Normal lung tissue contained  $2.604 \pm 0.729$  ng/mg TP of IL-8, as compared with  $8.499 \pm 2.327$  ng/mg TP for tumor samples ( $n = 52$ ). There were similar elevations of antigenic IL-8 for tumors of adenocarcinoma histology as compared with those of squamous cell phenotype,  $6.986 \pm 3.314$  ng/mg TP and  $9.752 \pm 2.956$  ng/mg TP, respectively.

**Immunohistochemistry Localizes IL-8 to Individual Tumor Cells.** We next used immunohistological localization to demonstrate the cellular origins of antigenic IL-8 in tumor tissue. Previous studies have convincingly demonstrated that human bronchogenic tumor cell lines may directly elaborate IL-8 (20, 24). We exploited immunohistochemical localization to identify cellular sources of the increased IL-8 in tumor tissue. Stains of tumor sections typically demonstrate heterogeneous tumor cell production of IL-8 in both adenocarcinomas and squamous cell carcinomas of the lung (Fig. 2). In addition, our results reveal that stromal cells within the local desmoplastic response may also serve as cellular sources for IL-8, especially in squamous cell carcinomas. It is important to note



**Figure 1.** IL-8 content of tissue samples of normal lung and bronchogenic lung tumors. Subgroup analysis by tumor histological diagnosis.

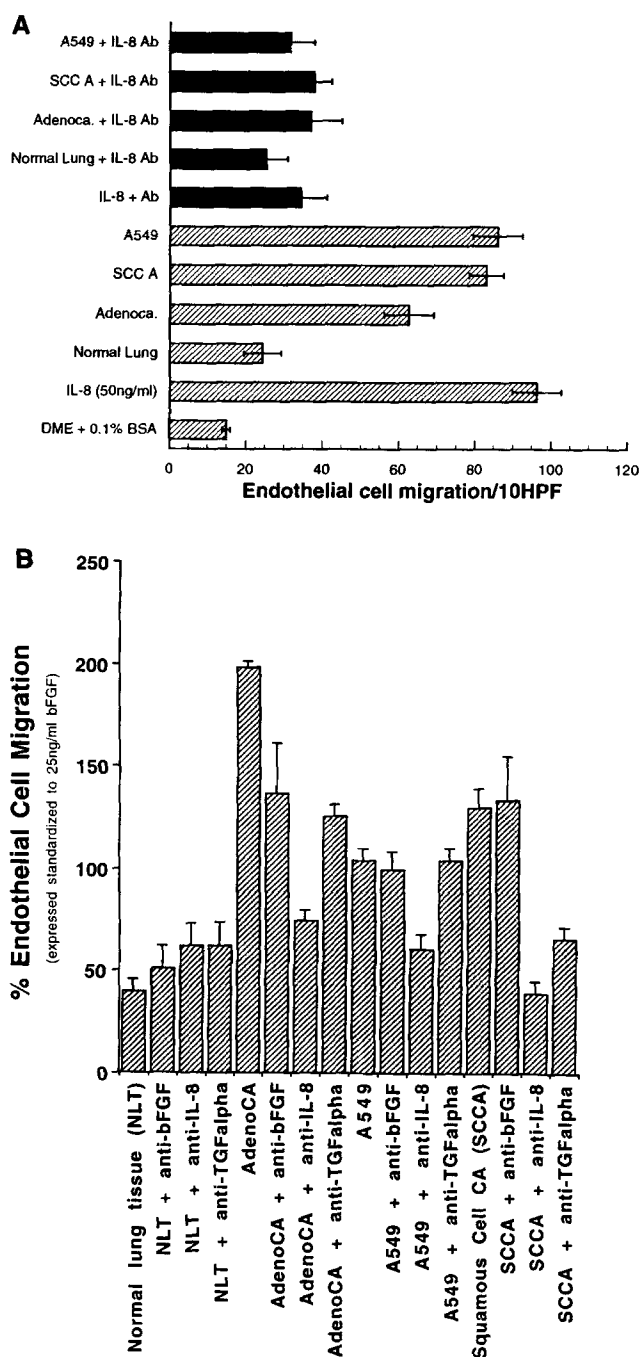
<sup>1</sup> Abbreviations used in this paper: BCE, bovine adrenal gland capillary endothelial cells; bFGF, basic fibroblast growth factor; TP, total protein.



**Figure 2.** Immunohistochemical staining of IL-8 in tumor tissue. (A) Normal lung with preimmune serum (200 $\times$ ). (B) Normal lung with anti-IL-8 (200 $\times$ ). (C) Squamous cell carcinoma with preimmune serum (200 $\times$ ). (D, E, and F) Squamous cell carcinoma with anti-IL-8 antibody (100, 200, and 400 $\times$ , respectively). (G) Adenocarcinoma with preimmune serum (200 $\times$ ). (H, I, J) Adenocarcinoma with anti-IL-8 antibody (100, 200, and 400 $\times$ , respectively).

that these specific findings may be reflected in the different clinical behaviors of squamous cell and adenocarcinomas. The more aggressive course of adenocarcinomas could result from its capacity to generate sufficient angiogenic signal by tumor IL-8 production, independent from contributions from the surrounding tissue response. The observation of a minimal inflammatory cell infiltrate in the tumor samples, despite the presence of significant quantities of IL-8, is unexpected and of particular interest.

**Endothelial Cell Chemotaxis Activity to Tumor Homogenates Is Blocked by Anti-IL-8.** To determine the relative contribution of IL-8 to the total angiogenic signal from bronchogenic carcinomas we employed a strategy using both in vitro and in vivo models of angiogenesis. First, concentrated tissue homogenates from normal lung, adenocarcinoma, and squamous cell carcinomas, as well as tissue extracts from suspensions of a bronchoalveolar carcinoma cell line (ATCC-A549; American Type Culture Collection, Rockville, MD), were evaluated for chemotactic activity toward BCE. Results for tissue samples were expressed as a percentage of chemotaxis activity induced by a standard of 50 ng/ml recombinant human IL-8. As illustrated in Fig. 3 A, samples from adenocarcinoma, squamous cell carcinoma, and A549 cells demonstrated 62, 84, and 86% respectively, of the maximal BCE chemotaxis induced by the IL-8 standard. BCE chemotaxis in response to media alone was 16% of the IL-8 standard. The addition of neutralizing antibodies to IL-8 reduce the endothelial cell chemotactic response to each of the test samples by ~66% (to a background level of 33% of the standard activity) (13, 20). Specifically, BCE chemotaxis in response to tissue samples plus anti-IL-8 was attenuated to similar background levels of 37, 39, and 32% of the standard, respectively, for adenocarcinoma, squamous cell carcinoma, and A549 tissue and cell homogenates. This data suggested that a significant portion of tumor-generated endothelial cell chemotaxis is mediated directly by IL-8. To further examine the angiogenic signal from tumor specimens in an attempt to characterize the relative contributions of other known angiogenic factors such as bFGF and TGF- $\alpha$ , a second series of experiments were performed. Endothelial cell chemotactic studies were repeated as before, with addition of neutralizing antibodies to either IL-8, bFGF, or TGF- $\alpha$ . Results are demonstrated in Fig. 3 B, standardized to the response generated from a standard of 25 ng/ml bFGF. Normal lung tissue generated only 40% of standard chemotactic activity, and was not significantly affected by the addition of any of the neutralizing antisera. Again, tumor samples alone generated brisk chemotactic responses, with 198, 130, and 104% standard activity, respectively, for samples of adenocarcinoma, squamous cell carcinoma, and A549 cell line tissue. The addition of neutralizing antibodies to IL-8 again resulted in significant reductions of chemotactic responses to tumor tissue, with decreases to 75, 39, and 61% standard activity, respectively, for adenocarcinoma, squamous cell carcinoma, and A549 samples. Anti-bFGF antibodies had no significant effect on responses to samples of A549 or squamous cell carcinoma tissue, but did yield a reduction in chemotactic activity of adenocarcinoma



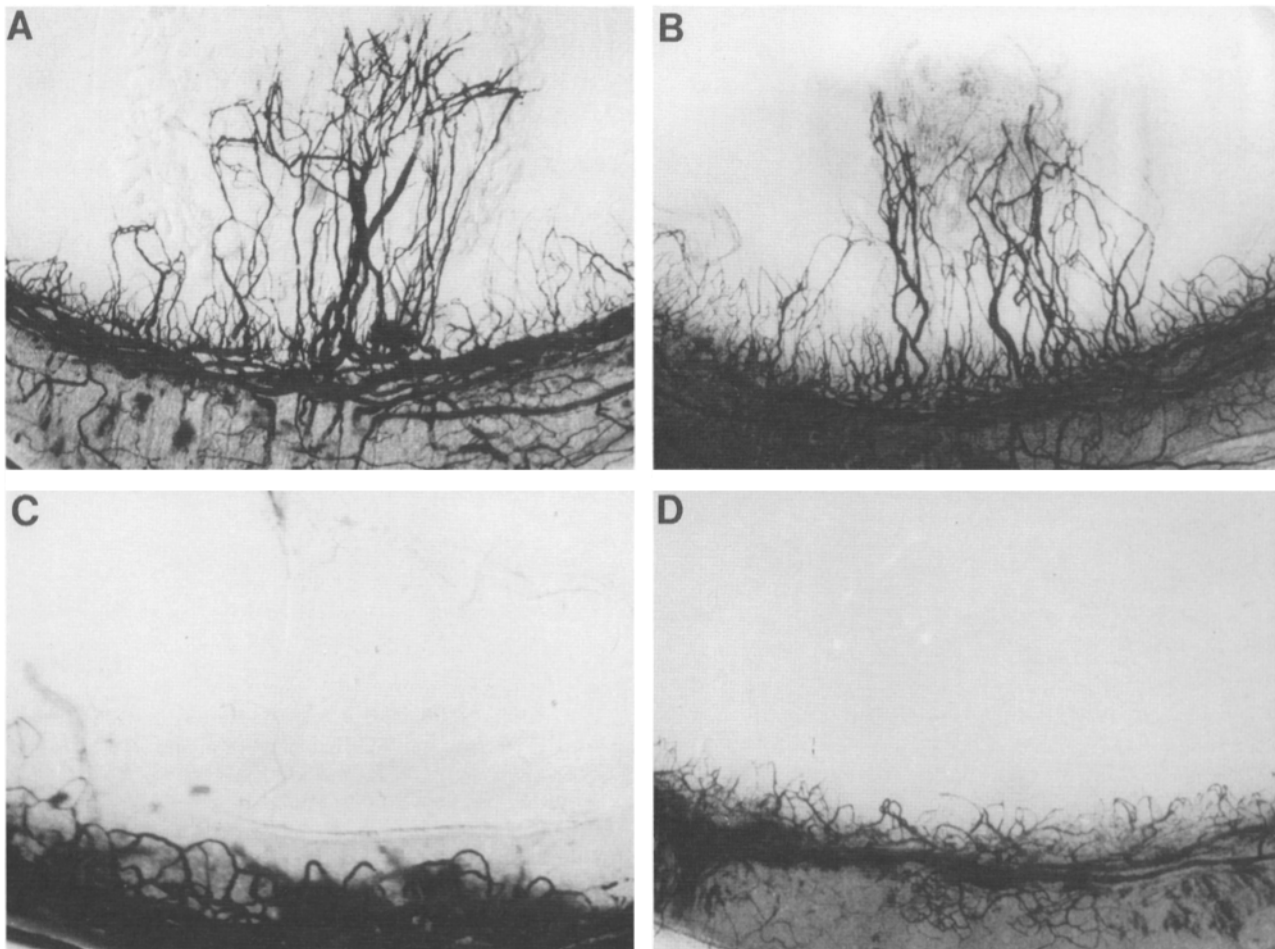
**Figure 3.** Endothelial cell chemotaxis. (A) Demonstrates responses standardized to 50 ng/ml IL-8, of specimens of normal lung, adenocarcinomas, squamous cell carcinomas, and A549 tissue in the presence or absence of specific neutralizing antisera to IL-8. Also demonstrates response to protein control. (B) Endothelial cell chemotactic responses to normal and tumor tissue, standardized to 25 ng/ml bFGF. Effect of addition of specific neutralizing antisera to IL-8, bFGF, and TGF- $\alpha$  is also demonstrated.

tissue from 198 to 137% standard activity. It is interesting to note that the neutralization of TGF- $\alpha$  effected no change in responses to adenocarcinoma or A549 tissue, but demonstrated a significant reduction in the chemotactic response

to squamous cell carcinoma tissue, from 130 to 71% standard activity.

**Neovascularization Induced by Tumor Homogenates Is Attenuated by Anti-IL-8.** We next evaluated the angiogenic signal mediated by tumor-associated IL-8 in an in vivo model. The previously well-characterized corneal micropocket model in the rat was employed (22, 23). Tissue samples and controls were incorporated into Hydrion (Interferon Sciences Inc., New Brunswick, NJ) pellets and then embedded into the normally avascular rat cornea (Fig. 4 and Table 1). Control samples of either 50 ng recombinant IL-8 or 50 ng recombinant bFGF induced positive corneal angiogenic responses of 80 and 100%, respectively. Samples from normal lung, adenocarcinoma, squamous cell carcinoma samples, and A549 cells produced positive angiogenic responses in 13 (1/8), 86 (6/7), 83 (5/6), and 100% (7/7), respectively. Anti-IL-8 antibodies completely abrogated the angiogenic response to IL-8 controls (zero of four corneas positive), but had no effect upon angiogenesis induced by bFGF (three of three corneas positive). The addition

of antibodies to IL-8 resulted in significant reduction of angiogenic responses to tumor tissue samples. A549 cell homogenates, which had yielded a 100% neovascularization rate, demonstrated no angiogenic activity in the presence of neutralizing antibodies to IL-8 (zero of five samples positive). Neutralizing antibodies to IL-8 caused a reduction in tumor sample angiogenesis of both adenocarcinoma and squamous cell carcinoma with 80% (four of five) and 80% (four of five) inhibition of angiogenesis, respectively. The addition of anti-IL-8 antibodies to normal lung samples did not induce a positive angiogenic response (zero of four positive), whereas normal lung samples with control antibodies had a 17% positive angiogenic response (one of six positive). It is important to note that there was no infiltration of the corneal tissue by inflammatory cells in any of the test samples or controls, suggesting that the angiogenic responses were mediated entirely by factors present in tumor tissue, rather than by any additional contributions from infiltrating inflammatory cell products.



**Figure 4.** In vivo angiogenesis demonstrating inhibition of lung tumor extract-induced corneal neovascularization by IL-8 neutralizing antibody. Representative photomicrographs of corneal neovascular responses. (A) Positive angiogenic response induced by A549 cellular homogenate. (B) Positive angiogenic response induced by tissue homogenate from human bronchogenic squamous cell carcinoma. (C) Negative angiogenic response from normal lung homogenate. (D) Markedly suppressed angiogenic response induced by squamous cell carcinoma tissue homogenate preincubated with neutralizing IL-8 antibody.

**Table 1.** *In Vivo* Angiogenesis

Test sample	Proportion of positive responses	
	(-) anti-IL-8 Ab	(+) anti-IL-8 Ab
Controls		
HBSS	0/3 (0)	0/4 (0)
IL-8 (50 ng)	4/5 (80)	0/4 (0)
bFGF (25 ng)	3/3 (100)	3/3 (100)
Lung Tissue		
Normal lung	1/6 (17)	0/4 (0)
Squamous cell carcinoma	2/3 (66)	1/5 (20)
Adenocarcinoma	3/4 (75)	1/5 (20)
A549 cell line	4/4 (100)	0/5 (0)

Composite of data demonstrating inhibition of lung tumor extract-induced corneal neovascularization by IL-8 neutralizing antibody.

These studies demonstrate that a primary angiogenic signal for bronchogenic carcinoma neovascularization is directly mediated by tumor-associated IL-8. Other previously described factors appear to also be involved to a lesser extent in tumor angiogenesis, with perhaps some variation by tumor histology. Although previous studies have demonstrated that various

tumors and tumor cell lines elaborate IL-8, the significance of tumor production of a neutrophil chemotaxin remained enigmatic (16, 20, 24-27). Our data, coupled with the recent characterizations of IL-8 bioactivity, suggest that tumor production of this potent angiogenic factor may be crucial for the neovascularization necessary for the initiation and maintenance of tumor growth. Interestingly, other investigations have noted increased angiogenic activity in hyperplastic lesions which precedes subsequent malignant transformation, suggesting that increased IL-8 production may play a functional role in, or possibly serve as a marker for, cytogenic dysregulation (28, 29). The observation of minimal inflammatory cell infiltrate, despite an appropriate chemotactic signal, remains perplexing and may reflect the attenuation of inflammatory cell chemotactic signal by other tumor-derived factors. Our recent description of the production of IL-1 receptor antagonist protein by human bronchogenic carcinoma lends support to this concept (30). Finally, as the demand for further vascular supply increases with continued tumor growth, successful maintenance of vascularization may require exploitation of normal inflammatory responses in order to provide adequate angiogenic signal. Accordingly, tumors may also elaborate additional factors to stimulate or enhance IL-8 production by other cells in a paracrine fashion, which may be reflected in the immunohistochemical localization of IL-8 in stromal cells within the tumor matrix. Future studies will explore the consequences of attenuation of tumor IL-8 production as a possible therapeutic intervention for bronchogenic carcinoma.

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