The Angiogenic Factor Interleukin 8 Is Induced in Non-Small Cell Lung Cancer/Pulmonary Fibroblast Cocultures

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Abstract

The interactions between tumor cells and surrounding stromal elements may promote the release of angiogenic factors. Although interleukin 8 (IL-8) is a major angiogenic factor in non-small cell lung cancer (NSCLC), the stromal contribution to IL-8 expression in primary NSCLC remains to be defined. To elucidate the role of stromal elements in NSCLC IL-8 production, normal pulmonary fibroblasts were cocultured with six representative NSCLC lines in direct and transwell assays. IL-8 transcripts and protein were consistently induced in fibroblasts and a subset of NSCLCs as a consequence of tumor/stromal coculture. In these cocultures, IL-8 was induced by IL-1α and an additional, as yet unidentified, soluble factor. These data underscore the importance of tumor/stromal interaction in the production of angiogenic peptides such as IL-8 in NSCLC.

Introduction

To progress beyond several millimeters in size, tumors must successfully recruit normal endothelial cells and develop a tumor vasculature (1). The recruitment of endothelial cells is promoted by soluble angiogenic factors, such as bFGF, VEGF, and IL-8 (1). Although these factors can be directly expressed by tumor cells, recent studies suggest that such angiogenic peptides can also be produced by surrounding normal stromal cells (2–4).

We have previously used a NSCLC/fibroblast coculture assay to demonstrate that tumor/stromal interactions promote the stromal-cell release of bFGF (5, 6). Whereas NSCLC and pulmonary fibroblasts that are cultured separately release no detectable bFGF, fibroblasts cocultured with NSCLC release readily detectable quantities of the peptide (5, 6). These results suggest that normal pulmonary fibroblasts may be an important microenvironmental source of angiogenic factors in NSCLC.

IL-8 is one of the most important angiogenic peptides in NSCLC (7–9). In recent studies, NSCLC cell lines such as A549 and Calu-1 were shown to produce IL-8 (7, 8). NSCLC-derived IL-8 stimulated the chemotaxis of endothelial cells and promoted angiogenesis in corneal pocket assays (7). IL-8 also enhanced the growth and angiogenesis of the two NSCLC cell lines in murine tumor models (8). In these assays, the administration of neutralizing IL-8 antibodies to tumor-bearing mice reduced NSCLC size by >40% and decreased tumor vascularity (8). However, the addition of recombinant IL-8 or neutralizing IL-8 antibodies did not alter the proliferation of NSCLC cell lines in vitro, suggesting that IL-8 had an indirect effect on tumor cell growth via its role in tumor angiogenesis (8). It was not possible to assess stromal IL-8 production in these murine tumor models because there is no identified murine IL-8 homologue (10).

In associated analyses of primary NSCLCs, the tumors were found to have ~4-fold more abundant IL-8 transcripts and protein than normal lung specimens (7). When primary NSCLC specimens were immunostained for IL-8, there was heterogeneous IL-8 expression in tumor cells. However, in certain NSCLCs, particularly squamous cell lung cancers, stromal cells also expressed increased IL-8 (7).

Tumor-derived cytokines stimulate the release of IL-8 by fibroblasts in vitro (11), raising the possibility that tumor/stromal interactions have similar effects on IL-8 release in vivo. To determine whether stromal cells produce IL-8 as a consequence of interaction with NSCLC cells, we have examined IL-8 expression in the tumor/fibroblast coculture model (5).

Materials and Methods

Cell Lines. Human fetal lung fibroblasts (ATCC CCL-153) and adult lung fibroblasts (ATCC CCL-210) were maintained in DMEM with 10% FCS. The A549 (bronchioloalveolar) and Calu-1 (squamous cell) NSCLC lines were maintained in DMEM/10% FCS, whereas SL-6 (large cell), H520 (squamous cell), H441 (adenocarcinoma), and H125 (adenosquamous) cell lines were maintained in RPMI/10% FCS. Media were supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mm l-glutamine. All cell lines were incubated at 37°C with 5% CO2.

Fibroblast/Tumor Cell Coculture. Fibroblasts were grown to confluence in six-well plates. NSCLC cells (2.5 × 105) were added either directly onto the fibroblasts or into the upper chamber of a transwell apparatus (Costar, Cambridge, MA), which physically separated the tumor cells from the fibroblasts but allowed for interaction between the cells via soluble factors. After an overnight culture in DMEM/10% FCS, cells were washed twice in serum-free medium and incubated for 24 h in 2 or 3 ml of serum-free DMEM. Selected cocultures were treated with neutralizing antibodies to IL-1α, IL-1β, TNF-α, or normal goat IgG at 1 μg/ml (R&D Systems, Inc., Minneapolis, MN), catalase (100 units/ml, Sigma), or NMA (0.75 mg/ml, Sigma). The conditioned media were harvested and centrifuged to remove cellular debris. Supernatants were frozen at −20°C until ELISA.

IL-8 ELISA. IL-8 protein levels in conditioned media were measured by ELISA (R&D Systems) per the manufacturer’s protocol. Briefly, 50 μl of sample or standard in 100 μl of diluent were added to each well coated with a murine monoclonal antibody against IL-8. To be within range, supernatants from A549 and H441 cultures were diluted 1:4 in serum-free DMEM. Other supernatants were analyzed undiluted. An IL-8 polyclonal antibody conjugated to horseradish peroxidase was added, and samples were incubated for 2.5 h. After six washes, substrate solution was added for 30 min. Absorbance was measured on a microplate reader (Bio-Rad) at 450 nm, and IL-8 concentration was determined in each sample by comparison to a standard curve that was generated by log-log plot (Bio-Rad software). Duplicate samples were analyzed for each coculture condition.

Northern Blot. Fibroblasts and tumor cells (2.5 × 105) were cocultured in 100-mm transwell dishes (Costar) under similar culture conditions described
IL-8 Release Is Enhanced in NSCLC/Stromal Cocultures. To determine whether IL-8 is induced as a consequence of tumor/stromal interaction, an IL-8 ELISA was performed on serum-free conditioned media from NSCLC cells (Fig. 1, T) and pulmonary fibroblasts (Fig. 1, F) cultured separately or together (Fig. 1, F/T). As described previously, the A549 NSCLC line constitutively expressed readily detectable levels of IL-8 protein, whereas the CCL-210 fibroblasts released negligible amounts of the chemokine (Fig. 1A, T, F). However, when A549 cells were cocultured with pulmonary fibroblasts, ~4 times more IL-8 was detected in the resulting conditioned medium (Fig. 1A, F/T).

To determine whether augmented IL-8 release was a common feature of NSCLC/stromal cocultures, five additional NSCLC cell lines (H441, Calu-1, SL6, H520, and H125) were also cocultured with CCL-210 fibroblasts (Fig. 1, B–F). The NSCLC cell line H441 also constitutively expressed moderate levels of the IL-8 protein (Fig. 1B), whereas the other NSCLC cell lines produced minimal quantities of the cytokine (Fig. 1, C–F). However, when all five additional NSCLC cell lines were cocultured with stromal cells, increased levels of IL-8 were detected in the resulting conditioned media (Fig. 1, B–F). Similar results were also obtained when the NSCLC cell lines were cocultured with another pulmonary fibroblast line (CCL-153, data not shown). Taken together, these data indicate that enhanced IL-8 release is a common feature of NSCLC/stromal cocultures.

IL-8 Release Does Not Require Direct Tumor/Stromal Cell Contact. To determine whether the increased IL-8 release in NSCLC/stromal cocultures requires physical contact between the tumor cells and fibroblasts, the A549 and Calu-1 cell lines were also cocultured with fibroblasts in a transwell apparatus that permits only the diffusion of soluble factors. As indicated in Fig. 2, A and B, IL-8 release was also augmented when either of two representative NSCLC cell lines (A549 or Calu-1) was cocultured with pulmonary fibroblasts in transwell.

Because the observed IL-8 release did not require direct tumor/stromal cell interaction, an IL-8 ELISA was performed on serum-free conditioned media from NSCLC cells (Fig. 1, T) and pulmonary fibroblasts (Fig. 1, F) cultured separately or together (Fig. 1, F/T). As described previously, the A549 NSCLC line constitutively expressed readily detectable levels of IL-8 protein, whereas the CCL-210 fibroblasts released negligible amounts of the chemokine (Fig. 1A, T, F). However, when A549 cells were cocultured with pulmonary fibroblasts, ~4 times more IL-8 was detected in the resulting conditioned medium (Fig. 1A, F/T).

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serum-free conditioned media were collected and assayed for IL-8 protein by ELISA. The are performed in the presence of the antioxidants, NMA, or catalase. Thereafter, 24-h antibodies directed against IL-1 had little effect on IL-8 induction (Fig. 3, B, C, and E). In contrast, the induction of IL-8 in Calu-1/stromal cocultures was specifically blocked by an IL-1α neutralizing antibody but was not inhibited by neutralizing antibodies to IL-1β or TNF (Fig. 3D). Similar results were obtained when Calu-1 was cocultured with either CCL-153 or CCL-210 pulmonary fibroblasts (Fig. 3D and data not shown).

To further explore the effects of IL-1α on IL-8 induction in Calu-1 NSCLC/stromal cocultures, additional cocultures were plated in transwell in the presence or absence of the neutralizing IL-1α antibody. Thereafter, RNAs from Calu-1 NSCLC cells alone, pulmonary fibroblasts alone, or transwell cocultures plated with or without the IL-1α antibody were prepared and analyzed for IL-8 transcripts. As indicated in Fig. 4, Calu-1/stromal coculture resulted in a 4-fold increase in IL-8 transcripts in the pulmonary fibroblasts and a 2-fold increase in IL-8 transcripts in Calu-1 cells. The addition of neutralizing IL-1α antibodies to the Calu-1/stromal cocultures reduced both IL-8 transcripts (Fig. 4A) and secreted protein (Fig. 4B) to baseline levels.

Taken together, these data indicate that IL-1α plays an important role in the induction of IL-8 in cocultures of fibroblasts with Calu-1 NSCLC cells. However, additional, as yet unidentified soluble factors induce or enhance IL-8 release in cocultures of pulmonary fibroblasts and the other examined NSCLC cell lines.

**Discussion**

In this study, IL-8 mRNA and protein are shown to be induced in pulmonary fibroblasts and certain NSCLC cell lines as a result of interaction between the two cell types. These observations underscore the importance of tumor/stromal interaction in producing angiogenic factors such as IL-8.

IL-8 directly promotes endothelial cell proliferation, chemotaxis, and tubular morphogenesis (14–16). The chemokine may also have indirect effects on angiogenesis resulting from the induction of MMP-2 and enhanced migration of endothelial cells through extracellular matrix (17). These observations are of particular interest because IL-8 is a major angiogenic factor in primary NSCLC tumors and murine models of NSCLC (7, 8).

![Figure 3: Characterization of the soluble factors mediating IL-8 induction in NSCLC/stromal cocultures](image)

**Fig. 3.** Characterization of the soluble factors mediating IL-8 induction in NSCLC/stromal cocultures. Fibroblasts (F) and NSCLC tumor cells (T; A, A549; B, SL6; C, H520; D, Calu-1; E, H125) were cultured alone or together (F/T). Tumor/stromal cocultures were also performed in the presence of neutralizing antibodies directed against IL-1α, IL-1β, or TNF-α or in the presence of control antibody. In addition, A549/fibroblast cocultures (A) were performed in the presence of the antioxidants, NMA, or catalase. Thereafter, 24-h serum-free conditioned media were collected and assayed for IL-8 protein by ELISA. The data are from a representative experiment. All experiments were performed independently 2–4 times.

stromal cell contact, the transwell cocultures were also used to determine which cell type produced increased quantities of the cytokine. In these experiments, tumor and stromal cells from transwell cocultures were separately lysed and analyzed for IL-8 transcripts (Fig. 2, C–F).

In transwell cocultures of A549 and pulmonary fibroblasts (CCL-210 (Fig. 2C) or CCL-153 (Fig. 2E)), IL-8 transcripts were significantly more abundant in fibroblasts cocultured with A549 than fibroblasts cultured alone; tumor-derived A549 IL-8 transcripts were abundant in both conditions (Fig. 2, C and E). In transwell cocultures of Calu-1 NSCLC cells and pulmonary fibroblasts (CCL-210 (Fig. 2D) or CCL-153 (Fig. 2F)), IL-8 transcripts were more abundant in both the NSCLC cells and the fibroblasts. Taken together, these data indicate that: (a) IL-8 transcripts are induced in pulmonary fibroblasts and certain NSCLC cell lines as a consequence of tumor/stromal interaction; and (b) the observed IL-8 induction did not require direct cell-cell contact, implicating a soluble mediator.

**Characterization of the Soluble Factor(s) Mediating IL-8 Induction in NSCLC/Stromal Coculture.** In previous studies, a variety of soluble factors, such as IL-1α, IL-1β, TNF-α, H2O2, and nitric oxide, have been shown to induce IL-8 in epithelial cells and fibroblasts (11–13). To assess the possibility that one of these factors was responsible for the IL-8 induction in tumor/stromal cocultures, the cocultures were plated in the presence or absence of neutralizing antibodies directed against IL-1α, IL-1β, or TNF or inhibitors of oxidant stress (NMA or catalase; Fig. 3). Neutralizing IL-1α, IL-1β, or TNF antibodies alone or in combination were unable to block the induction of IL-8 in A549/pulmonary fibroblast cocultures; inhibitors of oxidant stress (NMA and catalase) were similarly ineffective in blunting IL-8 release (Fig. 3A). In similar cocultures of SL-6, H520, and H125 NSCLC cell lines with pulmonary fibroblasts, neutralizing IL-1α, IL-1β, and TNF-α antibodies had little effect on IL-8 induction (Fig. 3, B, C, and E). In contrast, the induction of IL-8 in Calu-1/stromal cocultures was specifically blocked by an IL-1α neutralizing antibody but was not inhibited by neutralizing antibodies to IL-1β or TNF (Fig. 3D). Similar results were obtained when Calu-1 was cocultured with either CCL-153 or CCL-210 pulmonary fibroblasts (Fig. 3D and data not shown).

![Figure 4: IL-8 transcript and protein induction in Calu-1/fibroblast cocultures](image)

**Fig. 4.** IL-8 transcript and protein induction in Calu-1/fibroblast cocultures is inhibited by a neutralizing IL-1α antibody. Transwell cocultures of Calu-1 (T) and CCL-210 (F) were performed in 100-mm dishes for 24 h in serum-free medium in the absence (Transwell) or presence of neutralizing IL-1α antibody (Transwell + anti-IL-1α). A, total RNAs from each transwell sample or from fibroblasts (F) or tumor cells (T) cultured alone were size-fractionated, blotted, and probed with a [32P]IL-8 or [32P]glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) probe. B, serum-free conditioned media from the panel A fibroblasts (F) or Calu-1 (T) tumor cells cultured alone or transwell cocultures performed in absence (F/T) or presence of neutralizing IL-1α antibody (F/T + anti-IL-1α) were collected and analyzed for IL-8 protein by ELISA. The data are from one of two representative experiments.
In previous studies, NSCLC cell lines such as A549 were shown to produce IL-8 and to enhance angiogenesis in an IL-8 specific manner (7, 8). Although tumor-derived IL-8 clearly promoted angiogenesis in these in vitro assays and in vivo murine models, the contribution of stromal cells to IL-8 induction and production could not be examined in the model systems. That stromal cells might contribute to IL-8 production in NSCLC is of particular interest because pulmonary fibroblasts are the dominant source of IL-8 in other angiogenesis-dependent disease states, such as idiopathic pulmonary fibrosis (4).

In our own studies, two of six NSCLC cell lines (A549 and H441) expressed high basal levels of IL-8, whereas the remainder had low to negative IL-8 expression. These results are consistent with previous analyses in which 8 of 13 NSCLC cell lines produced high levels of IL-8 (18). Although only two of the NSCLC cell lines used in our studies had high basal levels of IL-8, all cocultures of NSCLC and pulmonary fibroblasts secreted increased amounts of the cytokine (Fig. 1). In representative tumor/stromal cocultures, IL-8 was always induced in pulmonary fibroblasts and was also induced in certain NSCLC cell lines (Fig. 2). These data raise the interesting possibility that pulmonary stroma may potentiate IL-8 production and related tumor angiogenesis in NSCLC.

These observations are consistent with recent studies suggesting a comparable stromal influence on tumor cell production of IL-8 in a melanoma nude mouse model (19). In these studies, IL-8 transcripts were significantly more abundant in melanoma cells metastatic to lung than in melanoma cells cultured alone in vitro. Furthermore, IL-8 transcripts were abundant in s.c. melanoma deposits but less abundant in liver metastases, suggesting that stromal effects on IL-8 may be organ dependent. Earlier studies have led to the assumption that the cancer cells are the source of IL-8 in primary tumor specimens. However, our data indicate that stromal cells produce increased IL-8 as well as enhance tumor cell IL-8 production in tumor/stromal cocultures. Tumor/stromal interactions have also been shown to influence the production of other angiogenic factors, such as VEGF and hepatocyte growth factor (HGF). For example, the VEGF promoter was strongly activated in tumor-associated fibroblasts in a murine model (2). Furthermore, HGF expression was increased in fibroblasts cocultured with tumor cells in additional analyses (3).

The mechanism by which IL-8 is induced in tumor/stromal cocultures remains to be defined. In several in vitro models, the induction of IL-8 required cell/cell contact (20, 21). Although IL-8 induction was most efficient in direct cocultures (Fig. 2), the cytokine was also induced when tumor cells were separated from stromal cells by a transwell membrane. These data implicate a soluble factor in IL-8 induction in the tumor/stromal cocultures. Antibodies directed against known soluble IL-8 mediators such as IL-1β and TNF-α, and inhibitors of oxidant stress did not blunt IL-8 release in the NSCLC/pulmonary fibroblast cocultures. Neutralizing IL-1α antibodies inhibited IL-8 induction in Calu-1/stromal cocultures but had no effect in cocultures containing other NSCLC cell lines. Taken together, these data suggest that additional as yet unidentified soluble factors mediate IL-8 induction in the majority of NSCLC/stromal cocultures.

Our results are consistent with mounting evidence that normal surrounding stromal cells may promote the growth and dissemination of tumors by modulating the release of critical angiogenic peptides. Further understanding of the interactions between tumor cells and adjacent normal stromal elements may lead to new therapeutic strategies.

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