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Anti-Vascular Endothelial Growth Factor Gene Therapy Attenuates Lung Injury and Fibrosis in Mice

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Vascular endothelial growth factor (VEGF) has important roles in endothelial cell proliferation, vascular permeability, and angiogenesis in a variety of inflammatory lesions. VEGF is expressed by various cells such as alveolar epithelial cells, Clara cells, macrophages, smooth muscle cells, and myofibroblasts in normal adult lungs. VEGF expression is upregulated by various stimuli, such as hypoxia, oxidative stress, and cytokines. VEGF binds to specific receptors, VEGFR1 (flt-1) and VEGFR2/KDR (flk-1), on endothelial cells. In addition, VEGF has been reported to be an indirect leukocyte migrating factor through inducing the expression of MCP-1 and IL-8.

The significance of VEGF in lung injury has previously been demonstrated. Overexpression of VEGF in the murine lung induced widespread intra-alveolar edema, suggesting that increased pulmonary vascular permeability in the early stages of acute lung injury may be induced, at least in part, by VEGF overexpression. VEGF was demonstrated to be unregulated in a LPS-induced acute lung injury model in mice, and the changes in balance between VEGF, angiopoietin-1, and angiopoietin-4 after LPS exposure may modulate neutrophil influx, protein leakage, and alveolar flooding in acute lung injury in mice. These results suggest important roles for VEGF in the vascular permeability and migration of inflammatory cells in lung injury.

Bleomycin-induced pulmonary fibrosis is an established model of lung injury and fibrosis. An imbalance between pro- and antiangiogenic activity in pulmonary fibrosis has been demonstrated in this model, and neoformation of blood vessels is associated with the rat lung fibrosis induced by bleomycin. To investigate the role of VEGF in lung injury and fibrosis using this model, we developed a transfection strategy that comprises transfection of the sflt-1 gene into skeletal muscles as a biofactory for anti-VEGF therapy in the lungs. We hypothesized that muscle cells infected with the sflt-1 gene would secrete sflt-1 protein into the circulating blood and that the sflt-1 protein would then capture VEGF on the target cells in the lung tissue, thereby blocking its signaling. On this basis, we examined the role of VEGF signaling in the development of bleomycin-induced pneumopathy in mice by assessing the vascular permeability, inflammatory cell infiltration, and von Willebrand factor expression in the lungs.

Materials and Methods

Model of bleomycin-induced pneumopathy

The present experiments were approved by the Committee on Ethics regarding Animal Experiments of Kyushu University Faculty of Medicine and were performed according to the guidelines of the American Physiological Society. C57BL/6 male mice (7–8 wk old) were purchased from KBT Oriental Japan and used in all experiments. The body weights of the mice were 20–25 g. The mice were anesthetized with an i.p. injection of pentobarbital sodium (1 mg/0.1 ml) and were perfused with a solution containing 1.5 U of heparin/kg body weight in sterile saline intratracheally. The lungs were dissected after perfusion, inflated with silicone rubber, and fixed in 10% buffered formalin. The left lung tissues were snap-frozen in liquid nitrogen and stored at −80°C until use.

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Abbreviations used in this paper: VEGF, vascular endothelial growth factor; sflt, solubleflt; BAL, bronchoalveolar lavage; BALF, BAL fluid; CTGF, connective tissue growth factor; IPF, idiopathic pulmonary fibrosis; ARDS, acute respiratory distress syndrome.

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**Determination of the VEGF kinetics in bleomycin-induced pulmonary fibrosis**

Frozen left lungs were homogenized in hypotonic buffer containing 25 mM HEPES, pH 7.5, 5 mM MgCl2, 1 mM EGTA, 1 mM PMSF, 1 mg/ml leupeptin, and 1 mg/ml aprotinin using a polytron homogenizer (Kinematica). The homogenates were centrifuged at 15,000 × g for 30 min at 4°C, and the supernatants were assayed for the VEGF kinetics by Western blotting analysis.

**Western blotting analysis**

The protein concentrations of the supernatants prepared above were determined using the Bio-Rad protein assay. Each supernatant was dissolved in sample buffer (133 mM Tris-HCl, pH 6.8, 0.1% SDS, 5% glycerol, 0.67% 2-ME, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) and boiled. In each lane of an SDS-PAGE gel, 30 μg of protein was electrophoresed, and the proteins were then transferred to polyvinylidene fluoride hydrophobic membranes (Millipore). The membranes were blocked with 5% nonfat dried milk in TBST buffer at 4°C for 2 h. The membranes were incubated with an anti-VEGF Ab (C-1; Santa Cruz Biotechnology), anti-ICAM-1 Ab (Santa Cruz Biotechnology), anti-von Willebrand factor Ab (Sigma-Aldrich), or anti-α-tubulin Ab in blocking buffer at 4°C overnight. After rinsing, the membranes were incubated with a biotinylated secondary Ab for 30 min at room temperature. The blots were developed using an ECL Western blotting detection kit (Amersham Biosciences). The blots were then scanned with an image scanner (GT-8700F; Epson). The relative band intensity was quantified using the NIH Image 1.61 software.

**Immunohistochemistry for VEGF and von Willebrand factor**

Paraffin sections (3-μm thick) were adhered to slides pretreated with poly-l-lysine. Following deparaffinization, immunohistochemistry was performed by a modified streptavidin-biotinylated peroxidase technique using a Histofine SAB-PO kit (Nichirei Corporation). Nonspecific protein staining was blocked with rabbit or goat serum for 30 min at room temperature. The sections were incubated with an anti-VEGF Ab (C-1; Santa Cruz Biotechnology) or an anti-von Willebrand factor Ab (Sigma-Aldrich) at 4°C overnight. For control incubations, the specific Abs were replaced by non-immune serum. The sections were then incubated with a biotinylated secondary Ab for 30 min, before treatment with 0.3% hydrogen peroxide in methanol for 30 min to inhibit any endogenous peroxidase activity. The slides were incubated with streptavidin-biotin-peroxidase complexes for 30 min and mounted.

**sflt-1 gene transfection**

The 3.3-kb mouse sflt-1 gene, originally obtained from a mouse lung DNA library, was cloned into the BamH1 (5’ ) and NotI (3’ ) sites of the eukaryotic expression vector plasmid cDNA3 (Invitrogen Life Technologies) as described previously (10). Mice were anesthetized with an i.p. injection of pentobarbital sodium (Schering-Plough) and in vivo electroproporation was performed as previously described (11). Briefly, the sflt-1 expression plasmid vector (50 μg/50 μl of saline) was injected into the femoral muscle using a 27-gauge needle. Immediately after the plasmid injection, a pair of electrode needles (Tokiwa Science) spaced 5 mm apart were inserted into the femoral muscle, one on each side of the injected site. Six 100-V square wave pulses (spaced 1 s apart) were applied with an electric pulse generator (CUY201; BTX), and the wound was closed. The mice were sacrificed at 1, 3, 5, 7, 10, and 14 days after the sflt-1 gene transfection, and whole blood was collected. The serum sflt-1 protein concentrations were assayed using a mouse soluble VEGFRI ELISA kit (R&D Systems). To assess the effect of the sflt-1 gene transfection on the early inflammatory phase and the late fibrotic phase in bleomycin-induced pulmonary fibrosis, the sflt-1 gene expression plasmid was administered at 3 days before or 7 days after the bleomycin instillation. For the negative control, empty vector pcDNA3 (Invitrogen Life Technologies) was administered at 3 days before or 7 days after the bleomycin instillation.

**Histopathological examination**

After thoracotomy, the pulmonary circulation was flushed with saline and the lungs were explored. The lung samples were fixed with 10% formalin overnight and embedded in paraffin. Paraffin sections (3-μm thick) were adhered to slides and stained with H&E. The pathological grade of inflammation and fibrosis in the whole area of the midsagittal section was evaluated by an experienced pathologist under 40× magnification and determined according to the following criteria: 0, no lung abnormality; 1, presence of inflammation and fibrosis involving <25% of the lung parenchyma; 2, lesions involving 25–50% of the lung; and 3, lesions involving >50% of the lung. Furthermore, the slides were stained with Sirius Red to assess the collagen deposition as previously described (12).

**Hydroxyproline assay**

Samples of the lung tissue were frozen in liquid nitrogen, lyophilized using a freeze-dry system (Labconco), weighed, minced into a fine homogeneous mixture, and hydrolyzed with 6 N HCl for 16 h at 120°C. The hydroxyproline content of each sample was determined according to the protocol of Woessner (13).

**DNA damage and apoptosis in lung tissues**

DNA damage and apoptosis were assessed by the TUNEL method using the DeadEnd fluorometric TUNEL kit (Promega) as previously described (14). The number of TUNEL-positive cells was counted in 20 randomly selected fields per section under a microscope at 200-fold magnification.

**Bronchoalveolar lavage fluid (BALF)**

A tracheotomy was performed in sacrificed mice. After insertion of a tracheal tube, the trachea was lavaged 5 times with 1-ml volumes of sterile saline at room temperature. The recovered fluids were filtered through a single layer of gauze to remove the mucus. The cells present in the lavage fluid were counted using a hemocytometer. Differential counts of BAL cells were performed on 200 cells stained with Diff-Quick (Baxter Diagnostics). The protein concentrations were determined using the Bio-Rad protein assay.

**ELISA for TNF-α and MIP-2 in BALF**

MIP-2 levels in BALF were measured with a cytokine-specific ELISA obtained from R&D Systems, and TNF-α levels were measured with ELISA obtained from BioSource International. For this assay, BALF was centrifuged, and supernatant was stored at −80°C until use for ELISA. The minimum detectable dose of MIP-2 was 1.5 pg/ml and TNF-α was 5 pg/ml.

**FIGURE 1.** Western blotting analysis for VEGF expression in lung homogenates after bleomycin instillation. Western blots (a) were scanned with an image scanner, and the relative band intensity was quantified using the NIH Image 1.61 software. Data are shown as the mean ± SEM of three mice (b). O.D., optical density. Significance was compared with mice at day 0 (*, p < 0.05).
Statistics

For statistical analysis regarding comparisons of the serum sflt-1 concentration, the number of cells and protein concentration in BALF, histological grade, the number of TUNEL-positive cells, hydroxyproline content, and results of ELISA, ANOVA followed by Scheffe’s F test was used. For comparison of the pathological grade, Kruskal-Wallis test followed by Mann-Whitney’s U test was used. Values of $p < 0.05$ were considered significant. Statistical analysis was performed with StatView J-4.5 (Abacus Concepts).

Results

Kinetics of VEGF in lung tissue after bleomycin administration

The VEGF concentration in the lung tissue homogenate was slightly increased at 1 day, and remarkably increased at 3–10 days after the bleomycin instillation. It was decreased at 14 days (Fig. 1). VEGF protein was detected in alveolar macrophages, type II alveolar epithelial cells and bronchiolar epithelial cells in untreated mice. The VEGF expression was up-regulated in these cells and the interstitium of inflammatory lesions at 3 to 14 days after the bleomycin instillation. von Willebrand factor expression was also increased along with the up-regulation of VEGF expression in inflammatory lesions (Fig. 2).

Kinetics of the serum sflt-1 concentration after gene transfection

sflt-1 was detectable in the serum of untreated mice, and remarkably increased at 3 to 7 days after the sflt-1 gene transfer (Fig. 3). Based on these findings, we injected the sflt-1 gene at 3 days before or 7 days after the bleomycin instillation to examine the significance of sflt-1 and VEGF in the early inflammatory phase (days 0–4) or late fibrotic phase (days 10–14) in this model.

Effect of sflt-1 gene transfer on the BAL

When the sflt-1 gene transfection was performed at 3 days before the bleomycin instillation, the protein concentration, but not the total cell count, was significantly decreased in the BALF at 7 days, whereas both the total cell count and the protein concentration were significantly decreased at 14 days compared with the values after bleomycin instillation alone (Fig. 4). The number of macrophages in percentages of total cell count was significantly increased, whereas that of lymphocytes was decreased compared with control mice at 7 and 14 days. That of neutrophils was significantly decreased at 14 days and tended to be decreased compared with control mice at 7 days (Table I). When the sflt-1 gene
transfection was performed at 7 days after the bleomycin instillation, both the total cell count and the protein concentration were significantly reduced at 14 days compared with the values after bleomycin instillation alone (Fig. 5). The number of macrophages in percentages of total cell count was significantly increased, whereas that of lymphocytes and neutrophils was significantly decreased compared with control mice at 14 days (Table 1).

**Effect of sflt-1 gene transfer on the histopathological findings**

The alveolar wall had begun to thicken with infiltration of neutrophils and lymphocytes at 7 days after the bleomycin instillation, compared with that of untreated mice. At 14 days after the bleomycin instillation, a large number of lymphocytes had infiltrated into the lung interstitium, and thickening of the alveolar septa, collapse of the alveolar spaces, and proliferation of fibroblasts were observed (Fig. 6A, a and b). The sflt-1 gene transfection at 3 days before or 7 days after the bleomycin instillation markedly attenuated the histopathological findings at 14 days (Fig. 6A, c–f, respectively). Empty vector pcDNA3 treatment at 3 days before or 7 days after the bleomycin instillation did not affect the histopathological findings at 14 days. Semiquantification of the histological analysis showed that sflt-1 gene transfer at 3 days before or 7 days after the bleomycin instillation significantly decreased the pathological grade at 14 days compared with controls (Fig. 6B). We found no abnormalities in lung tissues (n = 3 per each time point) at 1, 3, 7, 14, 21, and 28 days after sflt-1 gene transfection. These results are compatible with the results of other organs (15).

**Effect of sflt-1 gene transfer on lung fibrosis**

The sflt-1 gene transfer at either 3 days before (Fig. 7A, c and d) or 7 days after (Fig. 7A, e and f) the bleomycin instillation markedly decreased the positive staining for Sirius Red in the lung tissue at 14 days after the bleomycin instillation (Fig. 8A a). The sflt-1 gene transfer at either 3 days before and 7 days after the bleomycin instillation markedly reduced the number of TUNEL-positive cells at 14 days compared with controls (Fig. 8B a). The number of TUNEL-positive cells was significantly reduced these levels (Fig. 9A a). The results of Western blot analysis show that expressions of ICAM-1 and von Willebrand factor were increased these levels (Fig. 9A a).

**Cytokine measurement in BALF**

The results of cytokine measurement in BALF shows that the levels of TNF-α, and MIP-2 in BALF were significantly increased at days 7 and 14 after bleomycin instillation compared with those in saline-instilled mice. The sflt-1 gene transfer significantly decreased these levels (Fig. 9A a). The results of Western blot analysis show that expressions of ICAM-1 and von Willebrand factor were increased at 14 days after bleomycin instillation and decreased by the sflt-1 gene transfer (Fig. 9B a).
reduced the number of BAL cells, the expression of proinflammatory cytokines, the protein concentration, apoptosis, and pulmonary fibrosis. Gene transfer also attenuated von Willebrand factor expression in this model. Accordingly, VEGF may play an important role in the development of pulmonary fibrosis of this model, at least in part through increasing the vascular permeability, inflammation, and angiogenesis.

The detailed distribution of VEGF in this model has been reported (1). VEGF is present in alveolar type II cells, Clara cells, smooth muscle cells, and myofibroblasts in normal rat and human lungs. VEGF is predominantly localized in bronchiolar and

### Table I. The effect of gene transfer on the differentiation of BAL cells at day 7 and 14 days after bleomycin instillation

<table>
<thead>
<tr>
<th></th>
<th>M %</th>
<th>N %</th>
<th>Lym %</th>
<th>Eo %</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>94.0 ± 2.4</td>
<td>0.7 ± 0.2</td>
<td>5.4 ± 2.3</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Day 7 (n = 10 per each group)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>BLM</td>
<td>38.3 ± 9.4</td>
<td>17.2 ± 36.8</td>
<td>55.7 ± 7.2</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>BLM + pcDNA3 (d-3)</td>
<td>50.4 ± 8.1</td>
<td>10.8 ± 7.5</td>
<td>38.7 ± 11.8</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>BLM + sflt-1 (d-3)</td>
<td>66.0 ± 6.2*</td>
<td>5.6 ± 3.1</td>
<td>28.3 ± 5.8**</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>Day 14 (n = 10 per each group)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLM</td>
<td>44.2 ± 13.4</td>
<td>4.7 ± 2.6</td>
<td>50.9 ± 13.8</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>BLM + pcDNA3 (d-3)</td>
<td>42.0 ± 17.3</td>
<td>3.6 ± 2.2</td>
<td>54.4 ± 16.7</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>BLM + sflt-1 (d-3)</td>
<td>66.9 ± 8.8*</td>
<td>1.6 ± 1.0**</td>
<td>31.6 ± 8.6</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>BLM + pcDNA3 (d7)</td>
<td>43.5 ± 14.2</td>
<td>5.8 ± 5.1</td>
<td>50.5 ± 14.2**</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>BLM + sflt-1 (d7)</td>
<td>72.1 ± 10.6*</td>
<td>2.0 ± 1.9**</td>
<td>26.0 ± 9.8**</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

* BLM, bleomycin; d-3, transfected at 3 days before bleomycin instillation; d7, transfected at 7 days after bleomycin instillation.
* *p < 0.01, BLM + sflt-1 > BLM + pcDNA3; ** *p < 0.05, BLM + sflt-1 < BLM + pcDNA3.

**FIGURE 5.** Effect of sflt-1 gene transfer at 7 days after the bleomycin instillation on the number of BAL cells and the protein concentration at 14 days after the bleomycin instillation (*, *p < 0.01; **, *p < 0.05).**

**FIGURE 6.** Effect of sflt-1 gene transfer on histopathological findings. A. Representative results of the effect of sflt-1 gene transfer on the microscopic findings. Marked thickening of the alveolar septa, collapse of alveolar spaces, accumulation of a large number of lymphocytes in the alveolar walls, and proliferation of fibroblasts at day 14 after the bleomycin instillation (a and b). Sflt-1 gene transfer at 3 days before or 7 days after the bleomycin instillation attenuated the histopathological findings at 14 days after the bleomycin instillation (c, d and e, f, respectively). Original magnification, ×40 (a, c, and e) and ×400 (b, d, and f). B. Semiquantitative results of the effect of sflt-1 gene transfer on the pathological grade. Each circle or diamond corresponds to the data from one mouse (*, *p < 0.01).
alveolar epithelial cells and markedly increased in type II pneumocytes and myofibroblasts in fibrotic lung lesions. Interstitial fibrotic lesions were densely populated by VEGF-positive cells, but there were no positive signals for factor VIII staining in the fibrotic lesions at 28 days after bleomycin instillation (1). Accordingly, Fehrenbach et al. (1) suggested that VEGF exerts its action as a proinflammatory cytokine rather than an angiogenesis factor. However, the association of inflammatory cell infiltration with angiogenesis has been established. The angiogenesis process is known to be proinflammatory due to enhanced adhesion and permeability in inflammatory lesions (21). We also found that von Willebrand factor was expressed diffusely and more intensely on large vessels as well as small vessels in affected lesions than in

**FIGURE 7.** Effect of sflt-1 gene transfer on collagen deposition. A. Representative results of the effect of sflt-1 gene transfer on Sirius Red staining for collagen. Marked staining was seen in the peribronchiolar and alveolar walls in lung tissues at 14 days after the bleomycin instillation (a and b). Sflt-1 gene transfer at 3 days before or 7 days after the bleomycin instillation attenuated the positive findings at 14 days after the bleomycin instillation (c, d, and e, f, respectively). Original magnification, ×40 (a and c, and e) and ×400 (b, d, and f). B. Effect of sflt-1 gene transfer on the lung hydroxyproline content in bleomycin-induced pneumopathy in mice. Data represent the mean ± SEM of five mice (*, p < 0.01).

**FIGURE 8.** Effect of sflt-1 gene transfer on TUNEL staining. A. Effect of sflt-1 gene transfer on TUNEL staining in lung tissues after bleomycin instillation. TUNEL-positive cells in lung tissues at 14 days after the bleomycin instillation alone (a), transfected with pcDNA3 at 3 days before (b), transfected with sflt-1 at 3 days before (c), transfected with pcDNA3 at 7 days after (d), transfected with sflt-1 at 7 days after (e) after the bleomycin instillation. Original magnification, ×200. B. Effect of sflt-1 gene transfer on the number of TUNEL-positive cells in bleomycin-induced pneumopathy in mice. Data are shown as the mean ± SEM of six mice (*, p < 0.01).
non-affected areas. Although we did not directly measure the angiogenic activity, these results suggest that VEGF mediates inflammatory cell infiltration into the lungs associated with angiogenesis. We found an association between angiogenesis and inflammation in this model, which is earlier than that found in the study by Fehrenbach et al. (1). This may be the reason why we found an association between VEGF expression and von Willebrand factor in this model in contrast to the results of Fehrenbach et al. Recently, Inoki et al. (22) demonstrated that connective tissue growth factor (CTGF) binds to VEGF and inhibits VEGF-induced angiogenesis. CTGF and VEGF are downstream effectors of TGF-β, and these growth factors are known to be up-regulated in this model (23). At the advanced phase of fibrosis at 28 days, angiogenesis may be affected by other factors such as CTGF.

VEGF enhances the expression of chemokines MCP-1 and IL-8 on endothelial cells and in combination with IFN-γ synergistically induces endothelial cell production of the T cell chemoattractant IP-10 (24). In fact, IP-10 or neutralization of proangiogenic factor MIP-2 attenuates bleomycin-induced pulmonary fibrosis via inhibition of angiogenesis (8, 9). In addition, VEGF is a chemoattractant for mast cells, and mast cells can produce VEGF (25). Mast cells release fibrogenic mediators and stimulate fibroblast proliferation and collagen synthesis (26, 27). Melter et al. (28) demonstrated that ligation of CD40 on endothelial cells and monocytes by the CD40L on T lymphocytes potently induces VEGF production. Because VEGF links immune and inflammatory reactions, both early and late inhibition of VEGF can limit the development of fibrosis in this model.

The biological effects of VEGF are mediated by flt-1 and flk-1. Flk-1 is the major mediator of VEGF signaling, such as angiogenesis, vascular permeability, and endothelial cell survival, whereas flt-1 does not mediate these mitogenic signals but plays as a decoy receptor by preventing flk-1 from binding to VEGF (2). sflt-1 also binds to VEGF and inhibits its interaction with flk-1 (29). However, flt-1 mediates the migration of monocytes in response to VEGF (30). Fehrenbach et al. (31) demonstrated the expression of two VEGF receptors flt-1 and flk-1 in this model. Flt-1 was expressed in ciliated bronchial epithelium and type 2 pneumocytes, and flk-1 was expressed in Clara cells in normal mice lungs. This model was characterized by the accumulation of flk-1-positive mast cells in fibrotic lesions. Therefore, they suggested that VEGF/flk-1 represents the molecular link between proliferation of myofibroblasts and accumulation of mast cells (31). In consideration of these results, sflt-1 gene transfer prevented the development of this model through not only inhibiting the major biological function of VEGF mediated by flt-1, but also inhibiting the migration of monocytes mediated by flt-1 in this model.

As one of the complex mechanisms of lung injury and fibrosis, the importance of angiogenesis has been demonstrated in idiopathic pulmonary fibrosis (IPF) and acute respiratory distress syndrome (ARDS). IPF is defined as a specific form of chronic fibrosing interstitial pneumonia. Angiogenesis has previously been demonstrated, leading to precapillary-pulmonary anastomosis in fibrotic areas of IPF (32). The increased angiogenic activity in IPF has been attributed to an imbalance of proangiogenic and antiangiogenic chemokines (33). For vascular remodeling in IPF, ablation and redistribution of vessels have been demonstrated in the interstitial thickening, leading to gas exchange impairment (34). These studies suggest that aberrant vascular remodeling may play an important role in the pathogenesis of IPF (35).

In patients with ARDS, the plasma VEGF levels were elevated compared with control subjects, possibly contributing to abnormal capillary permeability in ARDS, and reduced in those who recovered (36). In contrast to the VEGF levels in the plasma, those in the epithelial lining fluid changed in the opposite direction with recovery from injury (37). Kaner et al. (38) suggested that alveolar epithelial cells are damaged in ARDS, possibly leading to the decreased levels of VEGF, because alveolar epithelial cells are the predominant source of VEGF (39). Accordingly, increased VEGF levels in the alveolar space may reflect recovery from ARDS (37). In contrast to these studies, Keane et al. (40) suggested that VEGF did not appear to contribute significantly to the angiogenic activity in ARDS, whereas CXC chemokines did. VEGF may indirectly enhance angiogenesis in inflammatory lesions through the up-regulation of chemokines in bleomycin-induced pneumopathy in mice.

In summary, many cytokines in lung tissues are known to increase VEGF production or potentiate its actions in bleomycin-induced pneumopathy. It is likely that VEGF itself also augments the inflammation through the up-regulation of cytokine expression and chemoattraction of inflammatory cells. Therefore, VEGF may have an important role to play in vascular permeability, inflammatory cell infiltration, fibrosis, and angiogenesis in this model. In vivo electroporation does not require the use of a viral vector or neutralizing antibody, and it is therefore possible to avoid problems regarding the pathogenicity of the viral vector or immune complexes. sflt-1 gene transfer may be a novel therapeutic strategy against lung injury and pulmonary fibrosis.
Disclosures

The authors have no financial conflict of interest.

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