Vascular Endothelial Growth Factor in Human Lung Transplantation*

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Study objectives: To determine levels of the vascular endothelial growth factor (VEGF) isoform consisting of 165 amino acids (VEGF165) in BAL fluid (BALF) from lung transplant recipients (LTXs).

Design: Bronchoscopy with BAL was performed on LTXs and normal volunteers (NVs).

Setting: University hospital.

Participants: LTXs (n = 57) and NVs (n = 15).

Measurements and result: VEGF165 concentrations in BALF were higher (mean ± SEM, 240 ± 32 pg/mL) for NVs (n = 15) vs 133 ± 14 pg/mL for LTXs (n = 37) who were stable without evidence of significant rejection or infection at 6 months after transplantation (p < 0.0001). BALF VEGF concentrations sampled at 24 to 48 h, 2 weeks, 4 weeks, and 6 months after transplantation for 11 LTXs who lacked rejection or infection at any time point were 71 ± 8 pg/mL, 80 ± 20 pg/mL, 82 ± 13 pg/mL, and 167 ± 31 pg/mL, respectively. VEGF concentrations in BALF for LTXs with cytomegalovirus (CMV) pneumonia were 55 ± 12 pg/mL (n = 10), 117 ± 33 pg/mL for grade A3 acute rejection (n = 9), and 82 ± 17 pg/mL (n = 14) for active bronchiolitis obliterans syndrome (BOS). Concentrations of VEGF in BALF at 6 months for the 32 stable recipients with bilateral lung transplantation were significantly higher for those with higher values for FEV1, and BALF VEGF concentrations were significantly lower in BALF at 6 months for those recipients who subsequently went on to develop BOS (86 ± 19 pg/mL) vs those who did not (158 ± 18 pg/mL; p = 0.03). Serum concentrations of VEGF did not correlate with VEGF concentrations in BALF, but serum VEGF was 291 ± 62 pg/mL at 10 to 14 days after transplantation vs 130 ± 20 pg/mL at 4 weeks for nine LTXs with paired samples (p < 0.02). Serum VEGF concentrations for NVs (n = 15) were 102 ± 15 pg/mL vs 94 ± 17 for stable LTXs (n = 12) at 24 weeks after transplantation and 123 ± 33 pg/mL for LTXs with active BOS (n = 10).

Conclusions: BALF VEGF concentrations are particularly depressed at early time points following lung transplantation, gradually improve in the absence of significant rejection or infection, and are lower with active rejection or CMV pneumonia.

Key words: BAL; lung transplantation; vascular endothelial growth factor

Abbreviations: BALF = BAL fluid; BLT = bilateral lung transplant; BOS = bronchiolitis obliterans syndrome; CF = cystic fibrosis; CMV = cytomegalovirus; LTX = lung transplant recipient; NV = normal volunteer; VEGF = vascular endothelial growth factor; VEGF165 = vascular endothelial growth factor isoform consisting of 165 amino acids

Vascular endothelial growth factor (VEGF) is a morphogenic cytokine that is highly expressed by lung epithelial cells1–3 and may play an important role in maintenance of the pulmonary vascular bed or responses to lung injury. This cytokine modulates embryonic angiogenesis and vasculogenesis by acting as a potent and essential mitogen for endothelial cells, and perturbed VEGF gene expression in the developing mammal has major consequences for embryonic development.4–6 Hyperexpression of VEGF in developing respiratory epithelium in embryonic transgenic mice disrupts morphogenesis and type I cell differentiation,7 and the presence of only a single functioning VEGF allele is lethal for the developing embryo.6 VEGF can also alter vessel permeability8 and appears to play an important role in wound healing and in maintenance of the differ-
VEGF can be produced by a variety of cell types in addition to epithelium, including macrophages, smooth muscle, mast cells, or neutrophils. Hypoxia, proinflammatory cytokines, estrogens, or androgens induce its expression, but hyperoxia, prednisone, or retinoids are inhibitory. Increased plasma or serum levels of VEGF have been detected in individuals with cancer or chronic inflammatory disease.

Endothelial growth factor gene expression by epithelial cells or other cell types as well as responses to angiogenic signals in the human lung are poorly characterized. High expression of VEGF by lung angiogenic signals in the human lung are poorly characterized. We hypothesized that VEGF levels in BAL fluid (BALF) may be altered during reparative processes after the newly transplanted human lung allograft sustains reperfusion injury or when the lung is undergoing rejection or cytomegalovirus (CMV) infection. Therefore, we measured the VEGF isoform containing 165 amino acids (VEGF165) in BALF and sera of 15 normal volunteers (NVs) and 57 lung transplant recipients (LTXs).

**Materials and Methods**

**Subjects**

The study population consisted of 57 LTXs and 15 NVs. All NVs were screened and recruited from the Madison, WI area and had unremarkable medical histories, normal findings on physical examination and spirometry, and no symptoms of an upper respiratory infection in the 4-week period prior to participation in the study. No NV had a history of tobacco smoking. All lung transplants were performed at the University of Wisconsin, and the group consisted of 34 patients with bilateral lung transplants (BLTs), 21 patients with single lung transplant recipients (NVs), and 57 lung transplant recipients (LTXs). Seventeen recipients had cystic fibrosis (CF), 22 had chronic obstructive pulmonary disease (COPD), 5 had idiopathic pulmonary fibrosis, 2 had sarcoidosis, 2 had Eisenmenger’s complex, 4 had α1-antitrypsin deficiency emphysema, 1 had lymphangioleiomyomatosis, 2 had bronchiectasis, 1 had hypersensitivity pneumonitis, and 1 had radiation fibrosis. Maintenance immunosuppression regimens consisted of cyclosporin A or tacrolimus plus azathioprine and prednisone. Histologic grading of acute rejection was performed according to Lung Rejection Study Group guidelines. Bronchiolitis obliterans syndrome (BOS) was diagnosed via lung biopsy using histologic criteria published by Yousen and coworkers and/or by a >20% decline from the best posttransplant FEV1 value that showed no improvement despite optimal immunosuppressive therapy. BALF from LTXs was collected at 6 months after transplantation. The study population consisted of 57 LTXs and 15 NVs. All study protocols were approved by the University of Wisconsin Human Subjects Committee, and informed written consent was obtained from all subjects. LTXs had BAL performed when undergoing surveillance or diagnostic bronchoscopy to rule out infection and/or rejection. BALF specimens were retrospectively identified for testing on the basis of clinical status and biopsy and BALF analysis.

Bronchoscopy was performed as previously described. Peripheral venous blood was sampled from all NVs and some LTXs just prior to bronchoscopy to obtain serum. A fiberoptic bronchoscope was wedged in a segmental bronchus of the right middle lobe or lingula, and four 40-mL aliquots of sterile, nonpyrogenic, isotonic sodium chloride solution were instilled through the bronchoscope and recovered immediately via gentle hand suction. Three 40-mL aliquots were used for the initial BAL at 24 to 48 h after transplantation for the 11 individuals with sequential BAL to minimize the risk of complications at this early time point. Pulmonary function testing was performed by registered pulmonary function technologists in the University of Wisconsin Pulmonary Function Laboratory.

**Processing of BALF**

The recovered lavage fluid aliquots were pooled, and cellular and fluid phases of the pooled lavage fluid were separated by centrifugation. Total cell counts were determined with a hemocytometer, and cytocentrifuge preparations were examined to obtain differential cell counts. BALF specimens were stored at −70°C until analyzed. Total protein concentration was determined via a modified Lowry method.

**Measurement of VEGF**

A commercial quantitative sandwich enzyme immunoassay (R&D Systems; Minneapolis, MN) was used to measure human VEGF165. This assay predominantly binds the monomeric VEGF165 but will also detect the VEGF isoform containing 121 amino acids. The assay utilizes a monoclonal anti-VEGF capture antibody, a polyclonal anti-VEGF detection antibody conjugated to horseradish peroxidase, and color development with tetramethylbenzidine/hydrogen peroxide. All assays were performed according to the protocol of the manufacturer, and the lower limit of detection was <10 pg/mL. Assays were performed on BALF aliquots that had only been thawed once. Some aliquots were reanalyzed after refreezing and rethawing to assess the effect of freeze-thawing on VEGF concentrations. The VEGF concentrations obtained after thawing were generally within 10% of the initial values. To minimize other sources of error, such as batch artifact, subject groups were intermingled on enzyme-linked immunosorbent assay plates and technicians were blinded to diagnoses and clinical situations.

**Statistical Analysis**

Data were analyzed on electronic spreadsheets (SuperCalc4; Computer Associates; San Jose, CA, and Excel; Microsoft; Redmond, WA) and database-statistics programs for microcomputers (Abstat 4.1; Anderson-Bell; Parker, CO, and SAS System; SAS Institute; Cary, NC). Values are expressed as mean ± SEM unless otherwise stated.

**Results**

BALF data were analyzed for VEGF cell concentrations and differential cell counts are given in Table 1. Concentrations of VEGF165 in BALF from LTXs (Fig 1) were determined at 6 months after transplan-
tation when the patients were clinically stable (no evidence of infection and with transbronchial lung biopsy histopathology revealing an acute rejection grade of A1 or A0). The mean value for VEGF (133 ± 14 pg/mL, n = 37) for these stable recipients was significantly lower than that of BALF from NVs (240 ± 32 pg/mL, n = 15; p < 0.0001). If normalized for protein content of BALF, these differences remained significantly lower for these stable LTXs at 6 months (1,268 ± 6 ng/μg) vs NV (3,334 ± 440 ng/μg; p < 0.0001). VEGF concentrations in BALF for LTXs with CMV pneumonia were 55 ± 12 pg/mL (299 ± 61 ng/μg, n = 10), 117 ± 33 pg/mL for grade A3 acute rejection (740 ± 255 ng/μg, n = 9), and 82 ± 17 (583 ± 150 ng/μg, n = 14) for active BOS; all were significantly decreased (p < 0.001) vs values for NVs, and mean concentrations for recipients with CMV pneumonia or BOS were significantly depressed compared to stable LTXs at 6 months (p < 0.05).

Concentrations of VEGF165 were determined for BALF sampled at 24 to 48 h, 2 weeks, 4 weeks, and 6 months after transplantation for 11 LTXs who were selected because they lacked significant rejection (grade ≤ A1) or infection at any time point (Fig 2). Mean VEGF concentrations were 71 ± 8 pg/mL at 24 to 48 h, 80 ± 20 pg/mL at 2 weeks, 82 ± 13 pg/mL at 4 weeks, and 167 ± 31 pg/mL at 6 months after transplantation. Concentrations of VEGF normalized to BALF total protein were 274 ± 79 ng/μg at 24 to 48 h, 596 ± 158 ng/μg at 2 weeks, 873 ± 234 ng/μg at 4 weeks, and 1,571 ± 390 ng/μg at 6 months. The VEGF concentrations in BALF at 6 months were significantly increased, compared to concentrations at earlier time points (p < 0.01 via two-tailed paired t test for 6 months vs 24 to 48 h, and p < 0.05 for 6 months vs 2 weeks). Concentrations of VEGF in BALF did not correlate with other BALF parameters (differential and total cell counts, total protein, total neutrophil concentration), nor did BALF VEGF concentrations for stable recipients at 6 months significantly vary by transplant indication (152 ± 21 pg/mL for CF, n = 14; 125 ± 31 pg/mL for emphysema, n = 13; 114 ± 34 pg/mL for interstitial disease, n = 5) or for single lung transplant vs BLT.

Although BALF VEGF concentrations also did

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**Table 1—BAL Analysis**

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>No.</th>
<th>Percent of Instilled Volume Retrieved</th>
<th>Cells/μL</th>
<th>Percent Macrophages</th>
<th>Percent Lymphocytes</th>
<th>Percent Neutrophils</th>
<th>Total Protein, μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stable LTXs</td>
<td>37</td>
<td>63 ± 1</td>
<td>257 ± 32</td>
<td>85 ± 2</td>
<td>9 ± 2</td>
<td>5 ± 1</td>
<td>133 ± 13</td>
</tr>
<tr>
<td>CMV pneumonia</td>
<td>10</td>
<td>65 ± 2</td>
<td>342 ± 103</td>
<td>75 ± 8</td>
<td>10 ± 2</td>
<td>24 ± 9</td>
<td>210 ± 32</td>
</tr>
<tr>
<td>Grade A3 rejection</td>
<td>9</td>
<td>61 ± 4</td>
<td>377 ± 103</td>
<td>67 ± 10</td>
<td>7 ± 2</td>
<td>24 ± 8</td>
<td>343 ± 112</td>
</tr>
<tr>
<td>BOS</td>
<td>14</td>
<td>57 ± 4</td>
<td>430 ± 103</td>
<td>70 ± 8</td>
<td>5 ± 1</td>
<td>24 ± 8</td>
<td>217 ± 36</td>
</tr>
<tr>
<td>NVs</td>
<td>15</td>
<td>68 ± 2</td>
<td>108 ± 11</td>
<td>90 ± 1</td>
<td>9 ± 1</td>
<td>1.4 ± 0.4</td>
<td>81 ± 11</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SEM.*
not correlate significantly with measures of lung function (FEV₁, FVC, diffusing capacity of the lung for carbon monoxide, total lung capacity, or diffusing capacity of the lung for carbon monoxide/alveolar volume) for BLT recipients who were stable at 6 months after transplantation, those with FEV₁ values > 85% of the normal predicted value at 6 months after transplantation had significantly higher BALF VEGF concentrations (145 ± 18 pg/mL, n = 18) vs those with FEV₁ percent predicted of < 85% (85 ± 14 pg/mL, n = 13) by independent two-tailed t test (p < 0.05). We also observed that BALF VEGF concentrations for stable individuals studied at 6 months were significantly lower for those who went on to develop BOS (86 ± 19 pg/mL, n = 11) vs those who did not (158 ± 18 pg/mL, n = 26; p = 0.03 by independent two-tailed t test) with subsequent follow-up from 2 to 8 years after transplantation (Fig 3).

Serum concentrations of VEGF₁₆₅ did not correlate with concentrations in BALF, but serum VEGF was elevated at 291 ± 62 pg/mL at 10 to 14 days after transplantation vs 130 ± 20 pg/mL at 4 weeks for nine LTXs with paired samples (p < 0.02 via two-tailed paired t test; Fig 4). Serum VEGF concentrations for NVs (102 ± 15 pg/mL, n = 15) were not significantly different from that for stable LTXs (94 ± 17 pg/mL, n = 12) at 6 months or LTXs with active BOS (123 ± 33 pg/mL, n = 10).

**DISCUSSION**

A variety of cells, including lung epithelial cells, express the VEGF gene, and a number of molecular species that differ in amino acid content due to alternative exon splicing can be expressed.⁹ The 206 or 189 amino acid isoforms are highly basic glycoproteins and are almost completely sequestered in the extracellular matrix but appear to be susceptible to proteolytic cleavage.²⁷ The smallest isoform (121 amino acids) is a freely soluble protein that binds heparin poorly. The 165 amino acid isoform that we measured in BALF is a 45,000-d glycoprotein that is secreted by cells and binds heparin. Although VEGF₁₆₅ is the major secreted isoform, a fair amount remains associated with the cell surface or the extracellular matrix. Proteolysis of the longer isoforms by plasmin has been demonstrated *in vitro* and is thought likely to occur *in vivo*, and heparin-binding ability closely correlates with mitogenic activity for vascular endothelial cells.²⁸

Unperturbed expression of VEGF may play a potentially important role in homeostasis and maintenance of the lung microvasculature.¹–³ Altered or attenuated VEGF gene expression by bronchoalveolar epithelial cells as a consequence of reperfusion injury, hyperoxia, or corticosteroid administration could affect lung allograft function and repair processes following implantation. Although proinflammatory stimuli can upregulate VEGF gene expression,¹⁶,²⁹ Demoly et al.³⁰ found that VEGF concentrations were not increased in BALF from patients with asthma or untreated chronic bronchitis when compared to control subjects. Induction of lung injury in the adult rat lung by hyperoxia is associated with the influx of inflammatory cells, but messenger RNA for VEGF and its receptors, *flt*-1 and *KDR/flk*, progressively decline in this model of lung injury.³¹ These authors have speculated that VEGF functions as a “survival factor” and maintains endothelial cells and prevents their death via apoptosis; a decline in VEGF gene expression under hyperoxic conditions may promote endothelial cell dysfunction and apoptosis. Numerous other investi-

![Figure 3](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21956/)

**Figure 3.** BALF VEGF concentrations at 6 months after transplantation for stable individuals who subsequently developed BOS (n = 11; closed triangles) vs those who did not (n = 26; open circles) and for NVs (closed circles).

![Figure 4](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21956/)

**Figure 4.** Serum concentrations of VEGF for nine allograft recipients at 2 weeks and 4 weeks after transplantation.
gations have demonstrated that VEGF promotes endothelial survival by inhibiting apoptosis. Additionally, Lassus et al. have shown that preterm infants who required surfactant replacement therapy had lower VEGF levels in tracheal aspirates compared to those who did not, and newborns with infant respiratory distress syndrome who went on to develop bronchopulmonary dysplasia failed to increase VEGF levels in tracheal aspirates when compared to those who did not develop bronchopulmonary dysplasia. These findings suggest that depressed VEGF expression may play an important role in the maturing fetal and newborn lung and in repair mechanisms following acute lung injury. Interestingly, VEGF concentrations in tracheal aspirate fluid did not correlate with concentrations in plasma in these neonates. These investigations suggest that VEGF concentrations in respiratory tract secretions may not be increased in the inflamed lung but may actually decline under certain conditions such as hyperoxia. We have found that VEGF concentrations are not increased in BALF from patients with CF or interstitial lung disease, compared to normal subjects. We also found that BALF VEGF concentrations were significantly decreased in idiopathic pulmonary fibrosis and decline with advancing age in normal subjects.

We found that concentrations of VEGF in BALF of LTXs were significantly depressed at early time points after transplantation but gradually increased with time. However, the mean VEGF concentration for stable LTXs 6 months after transplantation remained significantly lower than that for NVs and also were significantly lower if CMV pneumonitis or BOS were present. Vectorial secretion of VEGF onto lumenal surfaces has been reported for uterine endometrial epithelial cells, and vectorial intralumenal secretion may also occur in lung epithelium, as suggested by the observation that despite dilution of epithelial surface fluid by roughly 50-fold to 100-fold with isotonic saline solution via BAL, mean VEGF concentrations in BALF from normal individuals or LTXs were in the same range as VEGF concentrations in serum. Lassus et al. have also demonstrated that VEGF concentrations are higher in lower respiratory tract secretions than in peripheral blood.

We cannot rule out the possibility that VEGF levels in BALF merely reflect the amount of epithelial surface area lavaged or available for lavage in a given lung segment, rather than altered VEGF expression and secretion. Increased epithelial permeability may also promote the exit of VEGF from the airspaces, leading to the lower values we observed for LTXs. However, both hyperoxia and corticosteroids suppress VEGF gene expression, and the former may affect VEGF concentrations at early time points after transplantation, and the latter at both early and later time points after transplantation. We did not evaluate the expression of other VEGF isoforms such as the VEGF isoform with 189 amino acids, which is highly expressed in the lung, and perturbed expression of VEGF isoforms other than VEGF may have important effects on the lung vascular bed homeostasis and repair mechanisms following injury. Although VEGF concentration in BALF did not correlate with inflammatory indexes or with indexes of lung function for stable BLT recipients at 6 months after transplantation, we found it of interest that stable recipients with higher values for FEV1 percent predicted had a significantly higher VEGF concentrations in BALF and that BALF VEGF levels were lower for recipients who subsequently went on to develop BOS.

Additional research is required to determine whether VEGF gene expression plays a role in repair or maintenance of lung allograft microvasculature in LTXs. Vascular endothelial growth factor appears to prevent endothelial cell apoptosis in developing organisms, and decreased VEGF expression has been associated with endothelial cell apoptosis in mature tissue. Endothelial cell apoptosis during ex vivo preservation of the liver in animal models of hepatic transplantation appears to significantly depress function of liver transplants, and the development of arteriopathy in the transplanted heart has been linked to endothelial cell apoptosis. We speculate that sustained expression of VEGF is critical for preventing apoptosis of endothelial cells in the bronchial and alveolar circulations in the transplanted lung. We also speculate that application of VEGF to bronchial intralumenal surfaces may prevent endothelial cell death and promote repair processes in which angiogenesis plays a key role, particularly in areas of ischemic injury just distal to the allograft anastomoses where ischemic injury is often severe, and increases the risk of anastomotic dehiscence or the establishment of endobronchial aspergillosis. Application of VEGF to more distal airspaces or interventions to sustain its normal level of expression may help prevent injury to the microvascular bed in the newly transplanted lung and promote recovery from reperfusion injury after transplantation. The high partial pressures of oxygen in inhaled gas that are typically administered perioperatively and postoperatively to allograft recipients may have an adverse effect on the lung microvasculature by depressing VEGF gene expression and thereby promoting endothelial cell apoptosis.

In summary, VEGF levels appear to be depressed in BALF from LTXs when compared to NVs, particularly at early time points after lung
transplantation. A concentration gradient between epithelial secretions and serum suggests that vectorial intraluminal secretion may occur, creating a considerable concentration gradient for VEGF from airspaces to intravascular spaces. Animal models of lung injury and lung transplantation that examine the expression of VEGF isoforms in lung tissue as well as VEGF receptors such as flt-1 and flk-1, which appear to be expressed on mast and Clara cells as well as on endothelial cells, may provide information that advances our understanding of lung injury, repair, and microvessel homeostasis. Sustaining VEGF gene expression in the newly-transplanted lung or instilling it into airspaces may diminish reperfusion injury and improve posttransplant lung function.

ACKNOWLEDGMENT: We thank Professor Robert Auerbach for his advice and comments on the article.

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Clinical Investigations

142
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