Elevated Basic Fibroblast Growth Factor Levels in Patients With Pulmonary Arterial Hypertension*

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Study objectives: Cellular growth in the vascular wall, including endothelial and smooth-muscle cell proliferation, is recognized as a component of the obstructive vasculopathy observed in the small vessels of the lungs in pulmonary arterial hypertension (PAH). We hypothesized that angiogenic growth factors may have a role in the molecular mechanisms underlying this cellular proliferation.

Design: Case-control study.

Setting: Multicenter, tertiary care hospitals.

Participants: We studied 117 patients with PAH and 60 control subjects.

Measurements: We measured levels of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in the blood and urine of these subjects using an enzyme-linked immunoassay.

Results: Median levels of urinary and plasma bFGF were significantly higher in patients with PAH compared to normal control subjects. There was a difference in levels of urine and plasma bFGF according to etiology of pulmonary hypertension, with the highest levels seen in patients with primary pulmonary hypertension. Levels of urine or plasma VEGF were not significantly different between patients and control subjects.

Conclusion: Patients with PAH have substantial alterations in urine and plasma levels of bFGF. This molecule may have a role as a mitogenic factor in the endothelial and smooth-muscle cell proliferation seen in PAH.

Key words: angiogenesis; growth substances; pulmonary heart disease

Abbreviations: bFGF = basic fibroblast growth factor; CHD = congenital heart disease; CTD = connective tissue disease; IQR = interquartile range; PAH = pulmonary arterial hypertension; PAP = pulmonary arterial pressure; PPH = primary pulmonary hypertension; VEGF = vascular endothelial growth factor; WHO = World Health Organization

Pulmonary arterial hypertension (PAH) is a devastating illness characterized by a pulmonary vasculopathy that gives rise to an elevation in pulmonary vascular resistance. There has been considerable debate regarding the mechanisms underlying the development of PAH. Although initially focused on vasoconstriction and factors modulating vasomotor tone in the pulmonary circulation, it has more recently been proposed that cellular proliferation of endothelial and smooth-muscle cells is a more central component of the histopathologic changes seen in this disease. The search for molecular pathways...
that are involved in this cellular proliferation is therefore of particular interest.

Principles of angiogenesis have found application in a wide variety of disease states in clinical medicine. Vascular cellular proliferation is a key component of this process. Histologic evidence suggests that endothelial cell and smooth-muscle cell proliferation is present in the narrowed pulmonary vasculature affected in PAH, and this cellular proliferation is believed to contribute to the development and progression of this disease. Severe endothelial cell proliferation leading to obstruction of the pulmonary vascular bed without significant medial hypertrophy has also been described in PAH. Angiogenic growth factors may have a role in the mechanisms leading to the proliferation of these cells. The plexiform lesions, thin-walled vascular structures distal to sites of vascular obstruction, consisting largely of endothelial cells that express angiogenesis-related molecules, may represent a localized form of pathologic angiogenesis.

Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen and permeability factor. VEGF binds to at least two receptors, Flt-1 and Flk-1, expressed by endothelial cells. Flt-1 is associated with cell differentiation, whereas Flk-1 is thought to have a role in VEGF-mediated endothelial cell proliferation. Basic fibroblast growth factor (bFGF), stored in the extracellular matrix, has been documented to have a role in vascular cell migration, endothelial and smooth-muscle cell growth, and synthesis of extracellular matrix proteins. In view of their proliferative effects, these growth factors may have a pathophysiologic role in PAH. As both endothelial cell and smooth-muscle cell proliferation are present in PAH, bFGF may have a particular role because of its effects on both endothelial and smooth-muscle cells.

Although abnormal expression of bFGF and VEGF has been noted in animal models of pulmonary hypertension, their contribution to human PAH is not well understood. The detection of elevated growth factors in affected patients may help clarify the underlying mechanisms involved in the disease process. One area in which research on angiogenesis may have clinical application is the quantitation of angiogenesis. Quantitation of angiogenic proteins in body fluids has been used as an indirect measure of angiogenic growth factor activity in certain patients with tumors, as well as nonneoplastic diseases. We hypothesized that angiogenic growth factors may have a role in the cellular proliferation seen in the small vessels of the lung in PAH. We therefore measured bFGF and VEGF levels in the blood and urine of a large cohort of these patients.

Materials and Methods

Study Population

We studied a cohort of 117 patients with PAH (Table 1). The study was approved by the institutional review committee of the participating centers, and the patients gave informed consent. The control population consisted of 60 normal volunteers (median age, 37.5 ± 10.2 years [± SD]; female gender, 62%) from the participating centers without known cardiopulmonary disease or malignancy. Diagnosis of PAH was established by World Health Organization (WHO) criteria. Other causes of pulmonary hypertension were confirmed using patient histories, serologic testing, abdominal ultrasonographic findings, pulmonary function testing, lung/perfusion scintigraphy, echocardiography, chest CT, and cardiac catheterization, as appropriate. Covariates of interest including age, gender, etiology, WHO functional class, current medication, and hemodynamics were collected for each patient. Exclusion criteria included the following: age < 8 years old, pregnancy, neoplasia, pulmonary venous hypertension, pulmonary hypertension associated with disorders of the respiratory system and/or hypoxemia, and pulmonary hypertension due to chronic thromboembolic disease. Our procedures were in accordance with institutional guidelines for human subjects research.

Sample Collection and Assay

Blood samples were collected in a plasma ethylenediamine tetra-acetic tube. Samples were centrifuged at 2,500g for 10 min, and plasma was collected and stored at −80°C until it was assayed. Repeat freeze-thaw cycles were avoided. Spot urine specimens were obtained and stored at −80°C until they were assayed. Measurements of VEGF and bFGF were performed, by batch, in duplicate, using an enzyme-linked immunoassay for VEGF and bFGF (Quantikine; R&D Systems; Minneapolis, MN). The coefficients of variation for bFGF were 10% to 14% for plasma, and 9% to 11% for plasma. The coefficients of variation for VEGF were 4% to 7% for urine, and 5% to 7% for plasma. There was no significant cross-reactivity between the bFGF or VEGF antibodies and other known growth factors.

Statistical Analysis

Given the nonnormal distribution of the growth factor levels, statistical analyses were performed using nonparametric meth-

Table 1—Patient Characteristics (n = 117)*

<table>
<thead>
<tr>
<th>Etiology</th>
<th>PPH (n = 67)</th>
<th>CHD (n = 34)</th>
<th>CTD (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female gender</td>
<td>88</td>
<td>74</td>
<td>88</td>
</tr>
<tr>
<td>Age, yr†</td>
<td>44 (10–74)</td>
<td>33 (12–56)</td>
<td>52 (31–75)</td>
</tr>
<tr>
<td>Pulmonary vascular resistance, Wood units/m²</td>
<td>12 (3–37)</td>
<td>20 (5.2–31.6)</td>
<td>7 (2.2–21)</td>
</tr>
<tr>
<td>Cardiac output, L/min</td>
<td>4.5 (1.7–7.4)</td>
<td>4 (1.8–7.7)</td>
<td>4.8 (1.8–9.2)</td>
</tr>
<tr>
<td>Mean PAP, mm Hg†</td>
<td>53 (23–89)</td>
<td>71 (34–102)</td>
<td>54 (32–71)</td>
</tr>
<tr>
<td>Mean right atrial pressure, mm Hg</td>
<td>8 (1–25)</td>
<td>8 (4–19)</td>
<td>11 (1–18)</td>
</tr>
<tr>
<td>WHO class</td>
<td>3 (1–4)</td>
<td>2 (2–4)</td>
<td>3 (2–4)</td>
</tr>
<tr>
<td>Warfarin</td>
<td>59.4</td>
<td>66.7</td>
<td>55.5</td>
</tr>
<tr>
<td>Calcium-channel blocker†</td>
<td>31.0</td>
<td>18.2</td>
<td>41.7</td>
</tr>
<tr>
<td>Prostacyclin‡</td>
<td>65.2</td>
<td>25.8</td>
<td>71.4</td>
</tr>
</tbody>
</table>

*Data are presented as median (range) or %. †p < 0.05 for overall comparison between diagnostic groups.
ods, and summary measures are presented as medians and interquartile ranges (IQRs). The Wilcoxon rank-sum test was used to compare two-sample means, the Kruskal-Wallis test to compare means from multiple samples, and the Spearman rank correlation to examine correlation coefficients. Multiple comparisons were adjusted using the Bonferroni method. To perform regression analysis given the nonnormal distribution of the data, analysis of variance and regression on ranks of growth factor levels was used to adjust comparisons for multiple covariates.

Analyses were performed using SAS (version 8.0; SAS Institute, Cary, NC) and STATA (version 7.0; Stata Corporation; College Station, TX) statistical software packages. Statistical significance was defined as p ≤ 0.05.

Results

Urine bFGF Levels

The results of the growth factor levels by etiology of PAH are summarized in Table 2. Patients with PAH had significant elevations in median urine bFGF compared to control subjects (2,305 pg/L vs 1,111 pg/L, p = 0.0001). There was a difference in median urine bFGF level according to etiology of PAH: primary pulmonary hypertension (PPH), 2,741 pg/L; congenital heart disease (CHD), 2,330 pg/L; connective tissue disease (CTD), 1,493 pg/L; p = 0.15 (Fig 1). Significant pairwise comparisons in urine bFGF levels were observed for PPH vs control subjects (p < 0.0001) and for CHD vs control subjects (p < 0.05). There was no significant difference according to gender.

We examined the possibility that there may be a threshold level for urine bFGF. Using the 95th percentile in our control subjects as an upper limit of normal, we found that 21% of patients with PAH had elevations in urine bFGF. Twenty-six percent of patients with PPH, and 14% of patients with PAH-other (11% CHD and 19% CTD) had abnormally elevated urine bFGF levels (p = 0.008).

Plasma bFGF Levels

Median plasma levels of bFGF were significantly higher in patients with PAH than in control subjects (median, 1.9 pg/mL vs 0.5 pg/mL; p = 0.02). There was a difference in plasma bFGF levels based on etiology (PPH, 2.1 pg/mL; CHD, 1.7 pg/mL; CTD, 1.0 pg/mL; p = 0.3), but the only significant pairwise comparison was between PPH and control subjects (p = 0.05) [Fig 2, 3]. There was no significant difference between male and female patients.

We examined the possibility that there may be a threshold level for plasma bFGF. Using the 95th percentile in our control subjects as an upper limit of normal, we found that 51% of patients with PAH had elevations in plasma bFGF. Fifty-five percent of patients with PPH and 43% of patients with PAH-other had abnormally elevated plasma bFGF levels (p = 0.015).

Urine and Plasma VEGF Levels

There was no significant difference in median levels of urine VEGF (95 pg/mL vs 84 pg/mL, p = 0.9) between patients and control subjects. Overall, 7% of patients with PAH had elevated urine VEGF values (6% of patients with PPH and 8% of

Table 2—Angiogenic Growth Factor Levels in PAH*

<table>
<thead>
<tr>
<th>Etiology</th>
<th>Control Subjects</th>
<th>PPH</th>
<th>CHD</th>
<th>CTD</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine bFGF, pg/L</td>
<td>1,111 (592–1,834)</td>
<td>2,741†</td>
<td>2,330‡</td>
<td>1,493</td>
<td>≤ 0.0001</td>
</tr>
<tr>
<td>Plasma bFGF, pg/mL</td>
<td>0.5 (0.5–1.19)</td>
<td>2.15</td>
<td>1.74</td>
<td>1.04</td>
<td>0.05</td>
</tr>
<tr>
<td>Urine VEGF, pg/mL</td>
<td>84 (39–163)</td>
<td>100</td>
<td>92</td>
<td>71</td>
<td>0.68</td>
</tr>
<tr>
<td>Plasma VEGF, pg/mL</td>
<td>13.2 (7.5–42.3)</td>
<td>41.4</td>
<td>22.3</td>
<td>21.8</td>
<td>0.14</td>
</tr>
</tbody>
</table>

*Data are presented as median (IQR).
†Overall comparison of medians across diagnostic categories.
‡Significant pairwise comparison to control.
patients with PAH-other). Median levels of plasma VEGF were qualitatively higher in patients with PPH (41.4 pg/mL) than in those with PAH (21.8 pg/mL) or control subjects (13.2 pg/mL, p = 0.06). Overall, 7.5% of patients with PAH had elevated plasma VEGF values. Elevations in circulating VEGF were largely confined to the PPH group (12% of patients with PPH and 2% of patients with PAH-other). Differences were not significant within the subgroup of male vs female patients.

**Effect of Therapy on Angiogenic Growth Factor Levels**

The effect of medical therapy for PAH on growth factor levels was analyzed. Twelve patients (PPH, 6 patients; PAH-other, 6 patients) were not receiving prostanoid therapy, calcium-channel blockers, or endothelin blockers at the time of study (de novo patients). The median plasma bFGF level in this group of patients was significantly increased compared to control subjects (1.91 pg/mL, p = 0.02), as was the median urine bFGF level (2.338 pg/L, p = 0.002). The bFGF levels in these de novo patients were not significantly different from patients who were receiving specific therapy for PAH. Sixty-one percent of patients were receiving IV prostacyclin at the time samples were obtained for the cohort. Patients with PAH receiving prostacyclin had qualitatively higher median levels of plasma VEGF (42.9 pg/mL vs 25.4 pg/mL, p = 0.09) than did patients not receiving prostacyclin, although this did not reach statistical significance. This trend remained when analyzed for the PPH subgroup (48.1 pg/mL vs 28.8 pg/mL, p = 0.13). When adjusted for covariates associated with severity of disease, the effect of prostacyclin on plasma VEGF was diminished, and prostacyclin was not independently associated with altered growth factor levels. Calcium-channel blocker therapy or anticoagulation with warfarin did not affect growth factor levels.

**Correlation of Growth Factor Levels to Clinical and Hemodynamic Variables**

Levels of both bFGF and VEGF were analyzed for correlation to clinical and hemodynamic variables. In patients with PPH but not other forms of PAH, there was a modest correlation between mean pulmonary artery pressure (PAP) and plasma bFGF levels (r = 0.28, p = 0.04). We found a significant relationship between functional capacity and plasma bFGF in patients with PPH. In patients with poor functional capacity (WHO classes 3 or 4), median plasma bFGF was 4.2 pg/mL, compared to 0.5 pg/mL in patients with classes 1 or 2 (p < 0.003) [Fig 4].
When analyzed by bFGF level threshold, we found that patients with PPH and high plasma FGF levels had worse functional capacity ($p < 0.006$), but did not find any other significant associations to clinical or hemodynamic variables. We further examined the relationship between high plasma bFGF level and functional capacity in patients with PPH in a stepwise multivariable logistic regression model. We used the 95th percentile in our control subjects as a cutoff to delineate a high plasma bFGF threshold. We included clinical and hemodynamic covariates that had a univariate relationship ($p < 0.20$) to poor functional capacity to adjust for potential confounding in the model. High plasma bFGF level was independently associated with poor functional capacity (WHO classes 3–4) in patients with PPH (odds ratio, 7.09; 95% confidence interval, 1.9 to 26.8; $p = 0.004$).

Elevations in Both Plasma bFGF and VEGF

We examined a subgroup of 14 patients who had elevations in both circulating bFGF and VEGF. This subgroup consisted predominantly of patients with PPH ($n = 12; 86%; p \leq 0.001$). These 14 patients with PPH had higher mean PAPs and right atrial pressures ($p = 0.03$) and worse WHO functional classes ($p \leq 0.02$), compared to the PPH patients without elevations in both growth factors.

**Discussion**

The role of bFGF in PAH has been studied in animal models of pulmonary hypertension. Increased endogenous vascular elastase activity has been shown to occur in experimental models of PAH, and is associated with increases in bFGF production in the lung. As a result of elastase activity on the extracellular matrix, liberation of matrix-bound mitogens, including bFGF, occurs. bFGF levels increase progressively in airway, vascular, and gas exchange regions of monocrotaline-treated rat lungs. Examination of the pulmonary arterioles in humans with PAH has shown enhanced bFGF immunostaining in the endothelial and smooth-muscle cells, with highest levels observed in patients with PPH. With evidence that the lung is a site of significant bFGF production in PAH from both animal models and a human lung tissue study, plasma and urine measurements of bFGF, although they may not exactly parallel lung tissue levels, are likely to reflect spillover into the circulation from local production at the pulmonary microcirculation level. We did not see any relationship between bFGF level and cardiac index, suggesting that bFGF levels were not the result of a low cardiac output state. Unlike VEGF, bFGF has not been shown to be induced by hypoxia in vitro or in vivo. The analysis of angiogenesis-related transcription and growth factors in human skeletal muscle at different degrees of oxygen delivery has showed that bFGF messenger RNA transcription is not increased by hypoxia. It is therefore unlikely that the increased levels of bFGF observed in our study are due to decreased cardiac output and systemic hypoperfusion. Given the up-regulation of bFGF in the lung vasculature of patients with PAH, it is more likely that the elevated bFGF levels we observed were due to spillover from production at the lung vascular level. As well, matrix degradation, known to occur in PAH, may result in release of bFGF from matrix-bound stores.

The initial report from Stewart et al showed approximately a twofold elevation in endothelin-1 level in the venous plasma of patients with PAH, an abnormality of similar degree of magnitude when compared to the results of our findings with bFGF. Abnormally high levels of bFGF have been reported in the blood of 10% and in the urine of 37% of patients with cancer. The results of our study show that bFGF levels are significantly elevated in the
blood of 51% and in the urine of 21% of patients with PAH. Given the documented roles of bFGF in endothelial cell and smooth-muscle cell proliferation, bFGF may contribute as a growth factor to the cellular proliferation seen in the vascular bed in PAH.16

Our study shows differences in bFGF levels between the different diagnostic categories in PAH, with the highest levels seen in patients with PPH. In particular, we did not see significant elevations in bFGF levels in patients with CTD and PAH. One would expect that, given similar degrees of clinical and hemodynamic abnormalities between these diagnostic groups, we should have found similar levels of bFGF if this growth factor elevation was a secondary phenomenon. The fact that the bFGF levels were not similar suggests to us that the mechanisms leading to PAH in patients with PPH and other forms of PAH may not be the same. However, the small number of patients in the CTD group precludes us from making definitive conclusions with regard to bFGF levels in that population subgroup.

Our analysis of circulating blood and urine VEGF levels in patients with PAH did not show significant differences between patients and control subjects. Elevations in plasma VEGF were largely confined to the PPH subgroup. Patients with elevations in both bFGF and VEGF had worse clinical and hemodynamic profiles. Our study is limited in the interpretation of these VEGF levels; we measured plasma rather than serum VEGF, and this may have resulted in underestimation of circulating VEGF levels since VEGF can be stored in platelets. As well, several isoforms of VEGF are known to exist in lung (VEGF₁₈₉, VEGF₁₆₅, and VEGF₁₄₅), but not all are completely soluble, thereby limiting the extrapolation of circulating levels of VEGF to lung tissue levels of total VEGF. VEGF expression in humans with PAH has been localized to plexiform lesions.9 Plexiform lesions have been localized distal to sites of vascular obstruction. This is consistent with the hypothesis that plexiform lesions are an attempt at repair or neovascularization in advanced disease, and that VEGF overexpression may be part of the pathophysiology of these lesions.

This is the first report, to our knowledge, of elevated bFGF levels in the blood and urine of patients with PAH. The initial paradigm of vasoconstriction as disease mechanism in PAH and the search for vasoactive agents implicated in this disease gave rise to the finding of elevations in endothelin-1 levels in the blood of patients with PAH and subsequently to a novel target of therapy.17,18 With a shift in focus from vasoconstriction to abnormal vascular cellular growth in the small vessels of the lung, our data give support to the hypothesis that growth factors that stimulate abnormal cellular proliferation may contribute to the development of PAH. bFGF may be one such growth factor involved in the molecular mechanisms underlying endothelial and smooth-muscle cell proliferation in PAH. If supported by further data, modulation of bFGF may represent a novel target of therapy for PAH.

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