EG-VEGF, BV8, and their receptor expression in human bronchi and their modification in cystic fibrosis: Impact of CFTR mutation (delF508)

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Cystic fibrosis transmembrane conductance regulator (CFTR) protein dysfunction results in abnormal ion transport across the airway epithelium (7), progressively leading to the production of viscous mucus that obstructs the airways, underlying the subsequent inflammatory reactions and infections. Both processes combined with progressive bronchiectasis lead to the development of airway structural abnormalities that include subepithelial fibrosis, remodeling of airway epithelium and submucosal glands, and hyperplasia of airway smooth muscles (4, 31).

Airway remodeling in CF and other obstructive airway disorders is known to result from a long-standing bronchial inflammation (35, 39). Inflammatory mediators and growth factors have the capacity to cause cell infiltration and epithelial injury and to increase bronchial vascularization. The latter is due to the activation of the angiogenic process, which contributes to increase the portal entry for inflammatory cells (40). The magnitude of the vascular remodeling is correlated to the degree of inflammation resulting from infection, since it is not observed, to such extent, in noninfective chronic inflammation (40).

Signs of excessive angiogenesis have been reported in CF patients who develop highly vascularized nasal polyps requiring surgical resection. Moreover, angiogenesis-related complications, such as pulmonary hemorrhage and hemoptysis, are frequent in CF patients (11). Altogether these findings suggest that angiogenesis processes might contribute to the pathogenicity of CF. Nevertheless, the underlying angiogenic mechanisms associated with CF development and its aggravation are still unknown.

In CF patients, the increased pulmonary microvascular density associated with a significant increase in circulating vascular endothelial growth factor (VEGF) was first reported by Crawford et al. (8). The increase in the circulating VEGF levels was observed in subjects with CF and in subjects with other pulmonary inflammatory disorders. However, in CF, this increase was correlated to the level of bacterial infection and chronic airway inflammation (8).

Hence, elevations in circulating VEGF might promote pulmonary angiogenesis, which participates in the progression of the disease in the lung (27). The production of VEGF, and the induction of angiogenesis, is commonly interpreted as a consequence of the inflammation process. Importantly, Verhaeghe et al. (37) reported that local overproduction of proangiogenic factors in CF fetal human tracheal epithelial cells was sufficient to stimulate angiogenesis. Their results suggest that the intrin-


Table 1. Clinical characteristics of control and CF subjects

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<th>Control (n = 10)</th>
<th>CF (n = 10)</th>
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<tbody>
<tr>
<td>Male sex, n</td>
<td>7</td>
<td>8</td>
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<tr>
<td>Female sex, n</td>
<td>3</td>
<td>2</td>
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<tr>
<td>Age, yr</td>
<td>60–67 (10)</td>
<td>42 (1), 21 (6), 31 (3)</td>
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<tr>
<td>CFTR genotype, n</td>
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<td>F508del/F508del</td>
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<td>7</td>
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<tr>
<td>F508del/2184delA</td>
<td>0</td>
<td>2</td>
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<td>defF508/R798</td>
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<td>1</td>
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<tr>
<td>Bronchial colonization</td>
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<tr>
<td>Pseudomonas aeruginosa</td>
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n, No. of subjects; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator.

lic proangiogenic status of the CF respiratory epithelial cells contributes to the development of lung angiogenesis. Recently, Martin et al. (24) investigated the role of VEGF-A in the peribronchial pulmonary vascularization. They demonstrated that the airway epithelium is the main site of VEGF-A expression that was increased in CF patients and in Cfr-deficient mice. Also, they reported that the inhibition of CFTR activity induced a twofold increase in VEGF-A production. Altogether these data suggest that angiogenic factors might contribute the lung pathogenesis of CF.

During the last decade, two highly homologous cysteine-rich peptides, prokinetin 1 (PROK1) and prokinetin 2 (PROK2), were described as new angiogenic factors that are increased in response to stressful conditions such as hypoxia, inflammation, and abnormal vascularization (28). PROK1, also known as endocrine gland vascular endothelial growth factor (EG-VEGF), based on the functional similarities to VEGF, stimulates endocrine gland cell proliferation/migration and promotes angiogenesis in the mouse ovary, placenta, and cardiac tissue (2, 19). PROK2, or mammalian Bv8, is believed to affect behavioral circadian rhythm in the suprachiasmatic nucleus and to promote angiogenesis in the testis and cardiac tissue (6, 18). PROKs bind and activate two closely related G protein-coupled receptors, prokinetin receptor 1 (PROKR1) and 2 (PROKR2), which are 87% identical (21, 34). PROKs stimulate Ca2+ mobilization in PROK-expressing cells, presumably through a receptor/Gs protein interaction (21, 34). Pertussis toxin inhibits EG-VEGF-induced mitogen-activated protein kinase signaling (21), suggesting that EG-VEGF may also be coupled to Gs proteins. In addition, signal transduction studies showed that these ligands induce cAMP accumulation in PROK-expressing cells, indicating that PROKRs are also coupled to Gi proteins (5).

These peptides and their receptors were reported to localize in the brain, dorsal root ganglia neurons, granulocytes, placenta, and testis (12, 29). Yet the expression, localization, and potential role of EG-VEGF/Bv8 and their respective receptors in normal and CF lung angiogenesis are still unknown.

The present work aimed at determining the expression profiles of EG-VEGF/Bv8 and their receptors in the lung tissues from CF subjects compared with control nonsmokers. Because the airway epithelium appeared as an important site of EG-VEGF/Bv8 and PROKR expression in CF airways, we further studied the impact of CFTR chloride channel activity on EG-VEGF/Bv8 synthesis, secretion, and function in established normal and CF airway epithelial cells.
to the manufacturer’s protocol (RNAgents; Promega). Total RNA (1 μg) was used in reverse transcription using Superscript II-RNaseH reverse transcriptase under conditions recommended by the manufacturer (Invitrogen).

**Quantitative polymerase chain reaction.** BV8, BV8L, EG-VEGF, PROKR1, and PROKR2 mRNAs and 18S rRNA expressions were quantified by real-time PCR using a Light Cycler apparatus (Bio-Rad). The PCR was performed using the primers shown in Table 2.

**Fig. 1.** Expression of BV8, BV8L, and endocrine gland vascular endothelial growth factor (EG-VEGF) in human lung. A: RT-PCR detection of the ligand mRNA in normal and cystic fibrosis (CF) human airway epithelium. B–D: expression levels of EG-VEGF (B), BV8 (C), and BV8L (D) mRNAs were quantified by real-time RT-PCR and normalized to the 18S rRNA levels. Biopsies from nonsmoking normal (non-CF) and CF subjects were used. Data are expressed as means ± SE (n = 3 subjects). *Significantly different from the control (P < 0.05). NS, nonsignificant.

**Fig. 2.** BV8, BV8L, and EG-VEGF expression in human alveolar and bronchial epithelial cells. A–C: RNAs from alveolar (A459) and bronchial [human bronchiolar epithelial cell lines 16HBE14o- expressing wild-type cystic fibrosis transmembrane conductance regulator (HBE) and CFBE41o-(CFBE)] cells were extracted, and transcript expression levels of BV8, BV8L, and EG-VEGF were analyzed by real-time RT-PCR. Data are normalized by quantification of the 18S rRNA transcripts and are representative of at least 3 distinct experiments. D–F: quantification by ELISA of EG-VEGF production in supernatants (D), in protein extracts of A549, HBE, and CFBE cells (E), and in supernatants collected from apical and basolateral side of HBE and CFBE cells cultured on semipermeable filter (F). Data are expressed in pg/ml of conditioned media (E) and in pg/mg of protein extract. Data are expressed as means ± SE (n = 3). *Significantly different from the control (P < 0.05).
and SYBR green PCR core reagents according to the manufacturer’s instructions (Bio-Rad). The PCR condition and cycle were as follows: initial DNA denaturation 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, followed by an annealing step, and then extension at 60°C during 1 min. Each point was performed in triplicate. To ensure that the primers produced a single and specific PCR amplification product, a dissociation curve was performed during the PCR cycle, and only primers with a unique dissociation peak were selected, followed by migration on a 2% agarose gel to ensure that the PCR product was unique. The PCR products were then sequenced to confirm that the correct cDNA was amplified. The expression level of each gene was adjusted by the level of 18S mRNA and expressed as the ratio to 18S mRNA. The CFX Manager software was used to quantify levels of expression (Bio-Rad).

Electrophoresis and immunoblotting. Protein extracts, prepared as previously described (10), were subjected to an SDS-PAGE (12%) and immunoblot analysis using rabbit polyclonal anti-PROKR1 and -PROKR2 (Covalab), and monoclonal anti-β-actin (Hybridoma Bank, University of Iowa). Images were captured using the Molecular Imaging acquisition system (Vilber Lourmat).

Intracellular Ca$^{2+}$ mobilization assays. To evaluate intracellular Ca$^{2+}$ mobilization following BV8 or EG-VEGF stimulations, HBE and CFBE cells were seeded in poly-d-lysine-coated 96-well black wall tissue culture plates (BD Biosciences).

Ligand-stimulated Ca$^{2+}$ mobilization was assayed using fluo 3 Ca$^{2+}$ dye (Invitrogen) at a final concentration of 4 μM in dye loading buffer [DMEM F-12 medium without phenol red (Invitrogen) containing 2.5 mM probenecid] at room temperature for 1 h. Cells were then washed one time with dye loading buffer, and calcium mobilization in response to BV8 and EG-VEGF was assayed in a fluorometric imaging plate reader (Infinite M200; TECAN) as described previously (22).

Measurements of cAMP levels. For the measurement of cAMP mobilization, HBE and CFBE cells were plated into 12-well plates (1.5 × 10$^6$ cells/well). After 2 days of culture, cells were pretreated for 30 min with 0.5 mM 3-isobutyl,1-methylxanthine in serum-free medium before the addition of EG-VEGF or BV8 for 24 h. Intracellular cAMP concentrations were measured using the cAMP enzyme immunoassay system (GE Healthcare, Indianapolis, IN) as recommended by the manufacturer.

Measurement of the cAMP-stimulated CFTR activity. The plasma membrane cAMP-dependent halide conductance of HBE and CFBE cells was determined using iodide efflux as previously described (33). Iodide efflux was initiated by replacing the loading buffer with efflux medium (composed of 136 mM nitrate instead of chloride) and washing one time with dye loading buffer, and calcium mobilization in response to BV8 and EG-VEGF was assayed in a fluorometric imaging plate reader (Infinite M200; TECAN) as described previously (22).

**RESULTS**

CF tissues and CF cells differentially express and secrete BV8 and EG-VEGF. Previous gene expression profile analysis reported an upregulation of numerous proangiogenic genes in CF cells, namely basic fibroblast growth factor, fibroblast growth factor 5, placental growth factor, platelet-derived growth factor-C, platelet-derived growth factor-a polypeptide, angiopoietin 1, VEGF-A, and VEGF-C (37). However, the expression profiles of the new angiogenic factors, BV8 and EG-VEGF, has not yet been investigated. In CF lung tissue explants, both BV8 and EG-VEGF mRNA levels were upregulated compared with the control tissue (Fig. 1, A–C).

An alternatively spliced product of the BV8 gene encoding 21 additional amino acids compared with BV8, and designated as BV8L (for BV8 long form), has been shown to be broadly expressed. Compared with BV8, BV8L mRNA expression was shown to be lower in the brain, undetectable in the kidney, and higher in the lung and the spleen (5).

In CF, the mRNA level of BV8L did not show any significant changes compared with the control (Fig. 1, A and C), suggesting that BV8L has a limited role in CF.

At the cellular level, similar results were observed. The levels of EG-VEGF and BV8 mRNA were, respectively, approximately

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**Fig. 3.** Expression of prokineticin receptor (PROKR) 1 and 2 mRNA in normal and CF human lung. A and B: expression levels of PROKR1 (A) and PROKR2 (B) mRNAs were quantified by real-time RT-PCR and normalized to the 18S rRNA levels. Biopsies from non-CF and CF subjects were used. Data are expressed as means ± SD (n = 3). *Significantly different from the control (P < 0.05). C and D: mRNAs from alveolar (A549) and bronchial (HBE and CFBE) cells were extracted, and transcript expression levels of PROKR1 (C) and PROKR2 (D) were analyzed by real-time RT-PCR. Data are normalized by quantification of the 18S rRNA transcripts and are representative of at least 3 distinct experiments.
six and four times higher in CF cells (CFBE) compared with HBE cells (Fig. 2, A and B). Similar to the data obtained with the tissue analysis, no significant difference in BV8L mRNA levels could be observed between HBE and CFBE cells (Fig. 2C). There was no difference in the BV8L expression in bronchial and alveolar cells (Fig. 2C). The increase in EG-VEGF levels in CFBE cells was further investigated by comparing its secretion and content in A549, HBE, and CFBE cells using ELISA test. Figure 2, D and E, shows that both EG-VEGF secretion and content are significantly increased in CF cells. These data substantiate the findings at the mRNA levels and demonstrate that EG-VEGF secretion is also increased in CF cells. These results also suggest that CFTR protein might prompt EG-VEGF secretion. To get more insight into the site of EG-VEGF secretion by the polarized HBE and CFBE epithelial cells, apical and basolateral media were collected, and EG-VEGF were evaluated. The results indicated in Fig. 2F show that in both cells type EG-VEGF secretion was mainly apical.

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Fig. 4. Protein expression of PROKR1 and PROKR2 in normal and CF human lung. A and B: expression of PROK1 (A) and PROK2 (B) proteins in non-CF and CF human lung tissues and in HBE and CFBE cell lines was monitored with immunoblotting using polyclonal anti-PROKR1 and anti-PROKR2 antibodies, and the enhanced chemiluminescence detection system (ECL). Black arrowheads indicate the corresponding proteins (47 kDa). The blot is representative of 3 independent experiments. Expression of β-actin was monitored with monoclonal anti-β-actin antibodies. C and D: Image J quantification of the expression levels of the PROK1 (C) and PROK2 (D) protein standardized to β-actin expression. Data are expressed as means ± SE (n = 3). *Significantly different from the corresponding control (P < 0.05).

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Fig. 5. Representative photomicrographs of PROKR1 and PROKR2 immunostaining in human airways. Airway sections were obtained in human control patients (non-CF) vs. CF patients. Sections were stained with an antibody to PROK1 or PROK2 (brown color) and counterstained with hematoxylin and eosin. Photomicrographs were representative of results obtained in 6 controls and in 6 CF patients. Omission of primary antibodies or incubation with rabbit or mouse serum eliminated staining in control sections (undersized photographs). Scale bars 50 µm.
**PROKR1 and PROKR2 are highly expressed in CF.** BV8 and EG-VEGF share two cognate GPCRs receptors (PROKR1 and PROKR2) that share 80–90% identity (21, 34). We compared the levels of expression of the two receptors in CF and non-CF conditions. Using quantitative RT-PCR, only PROKR2 was highly expressed in CF tissues compared with normal lungs and compare their localizations in CF and non-CF lung tissues, we performed immunohistochemistry. Figure 5 shows strong staining could also be observed for PROKR2 in the apical membrane of the basal cells and within the goblet cells. No differences in the level of expression of PROKR1 and PROKR2 were observed when comparing bronchial with alveolar cells (Fig. 3, C and D).

At the protein level, with the use of Western blotting analysis, both receptors were upregulated in CF lung tissues and were retrieved at a band around 47 kDa (Fig. 3, A and B), as already described (29). Quantification of independent experiments indicated that PROKR1 and PROKR2 are highly expressed, not only in CF tissues, but also in CFBE cells (Fig. 4, A and B). PROKR1 and PROKR2 are expressed on the cell surface of respiratory epithelial cells. To get more insights into the cellular expression of PROKR1 and PROKR2 in the human lungs and compare their localizations in CF and non-CF lung tissues, we performed immunohistochemistry. Figure 5 shows that normal lung tissues express more PROKR2 than PROKR1. The intensities of expression for both receptors increased in the CF tissue sections. More importantly, in the CF condition, both receptors appear more concentrated at the apical membrane of the basal cells and within the goblet cells. Strong staining could also be observed for PROKR2 in the endothelial cells within the capillaries (Fig. 5). These findings suggest that PROKR1 and PROKR2 might play important roles in the respiratory functions both under physiological and pathological conditions.

**BV8 and EG-VEGF stimulate Ca2+ mobilization and cAMP production and activate chloride ion secretion.** BV8 and EG-VEGF have been reported to stimulate Ca2+ mobilization in PROKR-expressing cells (21, 34). We compared BV8 and EG-VEGF stimulation of Ca2+ mobilization in HBE and CFBE cells. Our results showed that both ligands significantly stimulate Ca2+ mobilization in HBE and CFBE cells (Fig. 6A). There was no difference in the levels of mobilized Ca2+ upon EG-VEGF and BV8 treatment in HBE and CFBE cells.

Because PROKR1 and PROKR2 activations were reported to stimulate cAMP accumulation, we examined their effects on cAMP accumulation in HBE and CFBE cells. In the absence of these ligands, only a basal cAMP production was detected. BV8 or EG-VEGF at 50 ng/ml significantly increased cAMP production in HBE. This increase was more important in the CFBE cells (Fig. 6B).

Consistent with the notion that cAMP accumulation accounts, at least in part, for the CFTR chloride channel activity, we evaluated the cAMP-activated anion conductance of the plasma membrane (Fig. 6C). As expected, CFBE cells did not show any iodine compared with HBE cells that endogenously express the wild-type CFTR (Fig. 6C). The treatment of HBE cells with BV8 or EG-VEGF showed a significant increase in the CFTR activity (Fig. 6D). These data suggest that EG-VEGF/BV8 through the activation of their receptors might play an important role in the physiological processes of normal and CF human lung function.
Role of CFTR-mediated chloride secretion on BV8/EG-VEGF transcription and secretion. We showed in Fig. 2 that BV8 and EG-VEGF expression and EG-VEGF secretion were higher in CF cells (CFBE) compared with normal cells (HBE). Data in Fig. 6 reporting the iodide-efflux results (Fig. 6D) confirmed the presence of a cAMP-activated chloride channel. Nevertheless, the relationship between CFTR activity and BV8/EG-VEGF transcription and secretion had still to be established. Using CFTR-inh172 (10 μM), a selective inhibitor of CFTR chloride channel activity (23), we observed an ~1.5-fold increase in EG-VEGF secretion in HBE cells (Fig. 7A).

To examine whether the effect of CFTR inhibition on EG-VEGF secretion was related to a transcriptional effect, we evaluated EG-VEGF, BV8, PROKR1, and PROKR2 mRNA expression using QPCR. Treatment of HBE cells with CFTR-inh172 (10 μM) induced a significant increase in EG-VEGF mRNA levels after 6 h of treatment (Fig. 7B).

The CFTR inhibition also increased BV8 and PROKR2 mRNA expression. We did not observe any significant effect on the mRNA expression levels of BV8L and PROKR1 (Fig. 7, C–F).

DISCUSSION

CF is the most common life-threatening inherited disease in the Caucasian population. Abnormal airway surface liquid results in recurrent lower respiratory tract infections, inflammation, and airway remodeling (4, 31). To our knowledge, this is the first report concerning the expression, localization, and potential role of two new angiogenic factors, BV8 and EG-VEGF, and their receptors, PROKR1 and PROKR2, in normal and CF human lungs.

Our results show that the airway epithelium is an important site for BV8, EG-VEGF, PROKR1, and PROKR2 expression. This expression was increased in CF patients compared with control subjects. In the airway epithelial cell line carrying a homozygote CF mutation (delF508), both ligands and their receptors were highly expressed compared with the wild-type cells. In these cells, the absence or the inhibition of CFTR chloride secretion resulted in increased BV8 and EG-VEGF biosynthesis and secretion. The present study also suggests that PROKR1 and PROKR2 are functionally coupled to the CFTR protein through the mobilization of intracellular cAMP.

BV8, EG-VEGF, PROKR1, and PROKR2 were localized to ciliated cells and in their precursors, i.e., the basal cells. The immunolabeling of both receptors showed that PROKR1 and PROKR2 were localized to the apical plasma membrane, where CFTR normally resides. Similar localization was also reported for the β2-adrenergic receptor (36). EG-VEGF was secreted at the apical site of CFBE and HBE cells, suggesting a potential role of prokineticins at the airway site.

The immunostaining study also revealed a cytoplasm localization for these receptors that probably corresponds to their constitutively internalization process.

Fig. 7. Time-dependent effect of CFTR inhibition on the expression of prokineticin factors and their receptors and on EG-VEGF secretion. A: effect of selective CFTR inhibitors on EG-VEGF secretion in HBE cells. Secretion of EG-VEGF was evaluated in conditioned media using ELISA following HBE cell treatment with 10 μM of CFTR-inh172 for the indicated times. B–F: effect of selective CFTR inhibitors on EG-VEGF (B), BV8 (C), BV8L (D), PROKR1 (E), and PROKR2 (F) expression in HBE cells. Cells were cultured in the absence or presence of 10 μM of CFTR-inh172. mRNA expression was measured using quantitative PCR, and the results are standardized to the housekeeping gene 18S. All results were expressed as means ± SE of 3 independent experiments.
In the epithelial cell model, both receptors were functional, and their stimulation by the corresponding ligands mobilized intracellular calcium and cAMP, as previously reported (21, 26).

Our results show that human bronchial epithelial cells carrying the homozygous CFTR mutation delF508 display a higher proangiogenic status compared with control epithelial cells. This proangiogenic status is characterized by an increased production of BV8, EG-VEGF, and PROKR2. For PROKR1 receptor, however, only its protein expression was upregulated in CF tissues and cells. These results suggest that both receptors are not regulated in the same way in normal and CF conditions. Our results suggest that BV8 and EG-VEGF should be considered as new angiogenic lung factors that might contribute to the increased angiogenesis observed in the CF disease.

Furthermore, these findings reveal that CF epithelial cells have the potential to efficiently induce angiogenesis through the release of multiple angiogenic factors, and raise the possibility that they could also initiate or increase the inflammation-related vascular remodeling in CF patients.

Our conclusion that CFTR dysfunction might trigger BV8 and EGVEGF synthesis is based on data obtained in cultured airway epithelial cells. Indeed, use of CF epithelial cells lacking apical chloride ion secretion or the inhibition of the CFTR activity in non-CF epithelial cells showed an increase in BV8/EG-VEGF biosynthesis and secretion.

Similar results were reported for the most potent angiogenic factor, VEGF-A. Verhaeghe et al. (37), using a human fetal cell line carrying the homozygous mutation delF508, and Martin et al. (24), by inhibiting the function of CFTR protein in a normal airway epithelial cell line, reported increased VEGF-A synthesis compared with control human cells. Our data provided circumstantial evidence of a role for CFTR dysfunction in BV8/EG-VEGF production. Finally, the absence of CFTR activity induced both increased BV8/EG-VEGF mRNA and protein synthesis, suggesting a transcriptional effect. Further studies are required to determine whether the effect is related to mRNA synthesis and/or mRNA stabilization.

The increase of BV8/EG-VEGF synthesis in CFBE cells was independent of infection and inflammation. However, increased BV8/EG-VEGF expression observed in CF airway epithelium at transplantation could be related to multiple factors, such as proinflammatory cytokines, microbial products from bacteria (116, 25), and to a hypoxic environment (15).

It is well established that hypoxic environment might accompany CF disease in the lung as a result of the CF-associated oxidative stress (1, 3). Hypoxia is also known to upregulate BV8 and EG-VEGF (19, 20), suggesting that the increased levels of both ligands observed in CF might be a consequence of this phenomenon.

Because BV8 and EG-VEGF can regulate a wide variety of biological processes, including angiogenesis, and inflammatory responses (20, 30), their elevated expression in CF may reflect the severity and neutrophil predominance of inflammation in this disorder (14).

Higher BV8 and EG-VEGF levels in CF may also be related to airway infection and that both ligands may be used as sensitive surrogate markers of airway inflammation associated with airway infection. Further studies including subjects with CF, chronic bronchitis, and bronchiectasis would further clarify the relationship between infection, inflammation, and BV8/EG-VEGF.

Our study highlights the ability of CF epithelial cells to intrinsically promote angiogenesis via the expression of angiogenic factors, including the EG-VEGF/Bv8 family. This suggests that therapies directly targeting a reduction in BV8/EG-VEGF synthesis and/or action to antagonized PROKR2 receptors could represent a promising method of reducing vascular remodeling in CF airways. Because angiogenesis is correlated with more severe pulmonary diseases, therapies based on the use of antiangiogenic factors could also be considered to reduce this phenomenon and to decrease the severity of the CF pathology.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.C., W.T., L.T., A.K., and M.B. performed experiments; S.C., W.T., L.T., and M.B. analyzed data; S.C. and M.B. interpreted results of experiments; S.C. and M.B. drafted manuscript; M.B. conception and design of research.

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