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

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\textbf{Running Head:} Upregulation of BV8 and EG-VEGF in cystic fibrosis.
Abstract
Enhanced lung angiogenesis has been reported in cystic fibrosis (CF). Recently, two highly homologous ligands, endocrine gland vascular endothelial growth factor (EG-VEGF) and mammalian Bv8 have been described as new angiogenic factors. Both ligands bind and activate two closely related G protein-coupled receptors, the prokineticin receptor (PROKR) 1 and 2. Yet, the expression, regulation and potential role of EG-VEGF, BV8 and their receptors in normal and CF lung are still unknown. The expression of the receptors and their ligands were examined using molecular, biochemical and immunocytochemistry analyses in lungs obtained from CF patients versus control and in normal and CF bronchial epithelial cells. CFTR activity was evaluated in relation to both ligands, and concentrations of EG-VEGF were measured by ELISA.
At the mRNA level, EG-VEGF, BV8 and PROKR2 gene expression was respectively ~5, ~4 and ~2 times higher in CF lungs compared to the controls. At the cellular level, both the ligands and their receptors showed elevated expressions in the CF condition. Similar results were observed at the protein level. The EG-VEGF secretion was apical and was ~2 times higher in CF compared to the normal epithelial cells. This secretion was increased following the inhibition of CFTR chloride channel activity. More importantly, EG-VEGF and BV8 increased the intracellular concentration of Ca^{2+} and cAMP, and stimulated CFTR-chloride channel activity. Altogether, these data suggest local roles for epithelial BV8 and EG-VEGF in the CF airways peribronchial vascular remodeling and highlighted the role of CFTR activity in both ligand biosynthesis and secretion.

Keywords: Cystic fibrosis, CFTR, lung angiogenesis, BV8/EG-VEGF, prokineticin receptors,
Introduction

Cystic fibrosis (CF) arises from mutations in the gene that encodes for the cystic fibrosis transmembrane conductance regulator (CFTR) protein (32), (9), an epithelial apical cAMP- and ATP-regulated chloride channel (38). The most frequent mutation, deletion of phenylalanine at position 508 (delF508) in the first nucleotide binding domain (NBD1), is detected in ~70% of CF chromosomes (13). The delF508-CFTR protein is misfolded, virtually completely blocked, and rapidly eliminated at the endoplasmic reticulum (ER) (17). 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 sub-epithelial 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 are known to result from a long-standing bronchial inflammation (39), (35). Inflammatory mediators and growth factors have the capacity to cause cell infiltration, epithelial injury, and to increase bronchial vascularization. The latter, is due to the activation of the angiogenic process, which contribute 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 as it is not observed, to such extent, in non-infective 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 to CF development and to its aggravation are still unknown.

In CF patients, the increased pulmonary microvascular density associated with a significant increase in circulating VEGF was first reported by Crawford et al. (8). The increase in the circulating VEGF levels was observed in subjects with CF as well as 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 participate 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 pro-
angiogenic factors in CF fetal human tracheal epithelial cells was sufficient to stimulate
angiogenesis. Their results suggest that the intrinsic pro-angiogenic 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 peri-bronchial 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, prokineticin 1
(PROK1) and prokineticin 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 glands
cell proliferation/migration and promotes angiogenesis in the mouse ovary, placenta and
cardiac tissue (19), (2). 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,
prokineticin receptor 1 (PROKR1) and 2 (PROKR2), which are 87% identical (21),(34).
PROKs stimulate Ca\textsuperscript{2+} mobilization in PROKR-expressing cells, presumably through a
receptor/Gq protein interaction (21),(34). Pertussis toxin (PTX) inhibits EG-VEGF-induced
mitogen-activated protein kinase signaling (21), suggesting that EG-VEGF may also be
coupled to Gi proteins. In addition, signal transduction studies showed that these ligands
induce cAMP accumulation in PROKR-expressing cells, indicating that PROKRs are also
coupled to Gs proteins (5).

These peptides and their receptors were reported to localize in the brain, dorsal root ganglia
(DRG) neurons, granulocytes, placenta and testis (29), (12). Yet the expression, localization,
regulation and potential role of EG-VEGF/BV8 and their respective receptors in the normal
and CF lungs 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 to control nonsmokers. Because the
airway epithelium appeared as important site of EG-VEGF/BV8 and PROKRs 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.

**Materials and Methods**

**Human tissues collection**

Peripheral human lung tissues (containing non cartilaginous airways) were obtained at the time of lungs transplantation from 10 nonsmoking CF adults, and from 10 nonsmoking controls undergoing lung resection for peripheral lung cancer (Grenoble Hospital). Clinical characteristics of these patients are provided in table 1. Lung tissue samples were fixed in 10% neutral buffered formalin by inflation-immersion and embedded in paraffin. To reduce the potential sampling bias related to irregular distribution of morphological abnormalities in CF patients, tissues were obtained from two to four blocks, whose locations were chosen randomly. In control subjects, only one block was available for the study. Collection and processing of human lungs were conformed to the Declaration of Helsinki and to all the rules of the local Committees on Human Research. Informed consent was obtained from each patient.

**Immunohistochemistry**

In this study, we also used ready 5-µm paraffin-embedded sections prepared from a human CF and non CF peripheral lung tissues. Slides were generously obtained from Pr. Lantuejoul, (Dept. of Pathology, DACP, Grenoble Hospital). Immunohistochemistry was performed as described previously (10). For antigen detection, sections were incubated with the following antibodies; anti-BV8, anti-EGVEGF, anti-PROKR1, and anti-PROKR2 (Covalab, France). Immunopositive staining was detected using a Vectastain ABC kit (Vector Labs), using DAB as the chromagen (Vector Labs). Slides were counterstained using hematoxylin and eosin (H&E) (Sigma Aldrich, France).

**Cell Culture**

We used human alveolar adenocarcinoma cell line; A549, and human bronchiolar epithelial cell lines: 16HBE14o-, expressing wild-type CFTR (HBE) and CFBE41o- (CFBE) derived from a cystic fibrosis patient, homozygous for the delF508 allele (delF508/delF508) (generous gift from Dieter Gruenert, University of California at San Francisco, CA). The cells were grown, respectively, in Dulbecco's MEM (DMEM) and in Eagle’s minimal essential medium (MEM) supplemented with 10% fetal bovine serum (Invitrogen, France), glutamine, penicillin and streptomycin at 37°C under 5% CO2 and routinely grown, respectively, in normal plastic dishes or in plastic dishes coated with an extracellular matrix containing fibronectin, collagen and bovine serum albumin.
To ensure the development of differentiated monolayers, some experiments were performed after culturing HBE and CFBE cells for 3–5 days at confluence on 12 mm Transwell polycarbonate filters (0.4-μm pore size). Differentiation of the epithelia was verified by the accumulation of zonula occludens-1 (ZO-1) at tight junctions, and by transepithelial electrical resistance measurement (data not show).

**Extraction of total RNA and reverse transcription**

Total RNA was extracted from lung tissues. The extraction was performed according to the manufacturer’s protocol (RNAgents; Promega, France). 1 µg of total RNA was used in reverse transcription using Superscript II-RnaseH reverse transcriptase under conditions recommended by the manufacturer (Invitrogen, France).

**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 (Biorad, France). The PCR was performed using the primers shown in table 2 and SYBR green PCR core reagents according to the manufacturer’s instructions (Biorad, France). 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 sec, follow 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 (Biorad, France).

**Electrophoresis and immunoblotting**

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

**Intracellular Ca²⁺ Mobilization Assays**

To evaluate intracellular Ca²⁺ mobilization flowing BV8 or EGVEGF 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, France) 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 1h. 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, Switzerland) 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 x 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 iodide). The extracellular medium was replaced every minute with efflux buffer (1 ml). After the steady state was reached, the intracellular cAMP level was raised by agonists (10 µM forskolin, 0.2 mM CTP-cAMP, and 0.2 mM isobutyl-methyl xanthine) to achieve maximal phosphorylation of the CFTR protein. The collection of the efflux medium resumed for an additional 6–9 min. The amount of iodide in each sample was determined with an iodide-selective electrode (Orion).

**EG-VEGF quantification by ELISA**

EG-VEGF secretion was measured by ELISA (PeproTech, France) in the cell culture conditioned media. Two separated standard curves were constructed to allow accurate readings of samples at upper and lower ranges of the assay. All samples were in the linear range of the standard curves. The detection limit of the assay was of 16 pg/ml. Data were expressed as pg/mg of protein extracted from HBE and CFBE.

**Data analysis**

Data are presented as the mean value ± standard error (SEM). Statistical comparisons were made using unpaired data Student’s t-test. Differences were considered significant when P < 0.05 (*P<0.05; **P<0.01; ***P<0.001).
Results

**CF tissues and CF cells differentially express and secrete BV8 and EG-VEGF**

Previous gene expression profile analysis reported an up-regulation of numerous pro-angiogenic genes in CF cells, namely basic fibroblast growth factor (bFGF), fibroblast growth factor 5, placental growth factor (PLGF), 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 as compared to the control tissue (Fig.1A, B and 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 to 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 to the control (Fig. 1A 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 ~6 and ~4 times higher in CF cells (CFBE) compared to HBE cells (Fig. 2A 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 2D and 2E show 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 figure 2F shows that in both cells type EG-VEGF secretion were meanly apical.

**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 to normal specimens (Fig. 3A and B).
However, both receptors were highly expressed in CF cells (CFBE) compared to normal one (HBE) (Fig. 3C and D). No differences in the level of expression of PROKR1 and PROKR2 were observed when comparing bronchial and alveolar cells (Fig. 3C and D).

At the protein level, using western blotting analysis, both receptors were upregulated in CF lung tissues and were retrieved at a band around 47 kDa (Fig. 3A 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. 4C and D).

**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 show 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 stimulates Ca\(^{2+}\) mobilization, cAMP production, and activates Cl\(^{-}\) secretion**

BV8 and EG-VEGF have been reported to stimulate Ca\(^{2+}\) mobilization in PROKR-expressing cells (21), (34). We compared BV8 and EG-VEGF stimulation of Ca\(^{2+}\) mobilization in HBE and CFBE cells. Our results showed that both ligands significantly stimulate Ca\(^{2+}\) mobilization in HBE and CFBE cells (Fig. 6A). There was no difference in the levels of mobilized Ca\(^{2+}\) upon EG-VEGF and BV8 treatment in HBE and CFBE cell. 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 50ng/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 to 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 lungs function.

**Role of CFTR-mediated chloride secretion on BV8/EG-VEGF transcription and secretion.**

We showed in figure 2 that BV8 and EG-VEGF expression and EG-VEGF secretion were higher in CF cells (CFBE) compared to normal cells (HBE). Data in figure 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 a ~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 (10µM) 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. 7C, D, E and 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, 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 as compared with control subjects. In the airway epithelium cell line carrying a homozygote CF mutation (delF508), both ligands and their receptors were highly expressed compared to 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 immunolabelling 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 which probably corresponds to their constitutively internalization process. 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 pro-angiogenic status compared to control epithelial cells. This pro-angiogenic 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, using CF epithelial cells lacking apical Cl− 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 factors, VEGF-A. Verhaeghe et al. (37), using human fetal cell line carrying the homozygous mutation delF508, and Martin et al. (24), by inhibiting the function of CFTR protein in 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 pro-inflammatory cytokines, microbial products from bacteria (25), (16), 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.

Since 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 EG-VEGF/Bv8 family. This suggest that therapies directly targeting a reduction in BV8/EG-VEGF synthesis and/or action to antagonized PROKR2 receptors could represent a promising way for reducing vascular remodeling in CF airways. Since angiogenesis is correlated with more severe pulmonary diseases, therapies based on the use of anti-angiogenic factors could also be considered to reduce this phenomenon and to decrease the severity of the CF pathology.

Acknowledgments

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References


**Figures Captations**

**Figure 1**

Expression of BV8, BV8L and EG-VEGF in human lung.

**Figure 2**

BV8, BV8L and EG-VEGF expression in human alveolar and bronchial epithelial cells.

**Figure 3**

Expression of PROKR1 and PROKR2 mRNA in normal and CF human lung.
PCR. Data are normalized by quantification of the 18S rRNA transcripts and are representative of at least three distinct experiments.

**Figure 4**
Protein expression of PROKR1 and PROKR2 in normal and CF human lung.
(A and B) The expression of PROK1 (A) and PROK2 (B) proteins in non-CF and CF human lung tissues, and in HBE and CFBE cell lines were 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 three 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 PROKR1(C) and PROKR2 (D) protein standardized to β-actin expression. Data are expressed as mean ± SE (n=3). Values with asterisk are significantly different from the corresponding control (P < 0.05).

**Figure 5**
Representative photomicrographs of PROKR1 and PROKR2 immunostaining in human airways. Airway sections were obtained in human control patients (non-CF) versus cystic fibrosis (CF) patients. Sections were stained with an antibody to PROK1 or PROK2 (brown color) and counterstained with haematoxylin 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.

**Figure 6**
(A) Evaluation of intracellular Ca\(^{2+}\) mobilization following BV8 and EG-VEGF stimulation in HBE and CFBE cells. Intracellular Ca\(^{2+}\) was measured as indicated in material and method.
(B) Stimulation of cAMP accumulation in HBE and CFBE cells following stimulation either with 50 ng BV8 or EG-VEGF. The accumulated cAMP was measured as indicated in material and method section. Data are expressed as mean ± SE (n=3). Values overwritten with different letters (a, b, c) are significantly different from each other (P < 0.05).
(C and D) CFTR activity was monitored using cAMP-stimulated iodide release from CFBE and HBE cells. The iodide efflux was measured after 1 and 2 min of cAMP-dependent protein kinase stimulation (C) or after 5 min stimulation by 50 ng of BV8 or EG-VEGF (D). Iodide release from CFBE and HBE cells are expressed as nmoles/min (mean ± S.E., n=4).

**Figure 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 test following HBE cells treatment with 10 µM of CFTR-inh172 for the indicated times.
(B-C) Effect of selective CFTR inhibitors on EG-VEGF, BV8, BV8L, PROKR1 and PROKR2 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 the results were expressed as mean ± SEM of 3 independent experiments.
Tables

**Table 1:** Clinical characteristics of Control and CF subjects.

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**Table 2:** Primers used for real-time (RT) PCR. FW: Forward, BW: Backward (reverse primer).

<table>
<thead>
<tr>
<th>ADNc</th>
<th>Primer Sequence 5’ → 3’</th>
<th>Size (pb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG-VEGF</td>
<td>FW : AGGTCCCTCTCTTGAAACG</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td>BW : TCCAGGCTTGCTCAAGGAAG</td>
<td></td>
</tr>
<tr>
<td>Bv8</td>
<td>FW: TGTGACAAGGACTCDSAATGCTTGA</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>BW: CCGCCAAAATAATGAATCTTTACG</td>
<td></td>
</tr>
<tr>
<td>Bv8L</td>
<td>FW: CTATGGGAACACTGGGAGAC</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>BW: AGACATGGGCAAGTGATG</td>
<td></td>
</tr>
<tr>
<td>PROKR1</td>
<td>FW: CCGCATGGGAAACTTCATCT</td>
<td>338</td>
</tr>
<tr>
<td></td>
<td>BW: GTGCGCATCTGCTGACAGA</td>
<td></td>
</tr>
<tr>
<td>PROKR2</td>
<td>FW: CACCATGACCTGCTGACAGA</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>BW: ACAGTGGGCAAAGTGACAGA</td>
<td></td>
</tr>
<tr>
<td>18S</td>
<td>FW: AAACGGCTACCATCAAGA</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>BW: CCTCAATGGATCTCGTCA</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2

A. EG-VEGF content (pg/mg)

B. BV8/18S/1011

C. BV8L/18S/1013

D. EG-VEGF secretion (pg/ml)

E. EG-VEGF content (pg/mg)

F. EG-VEGF secretion (pg/ml)
Figure 3

A  PROKR1

B  PROKR2

C  PROKR1

D  PROKR2
**Figure 4**

**A**

α-PROKR1

α-β-actin

Non-CF | CF
---|---
47 | 47
40 | 40

**B**

α-PROKR2

α-β-actin

Non-CF | CF
---|---
47 | 47
40 | 40

**C**

PROKR1/actin proteins expression (A.U)

Non-CF | CF | HBE | CfBE
---|---|---|---
0.5 | 2.0 | 1.0 | 1.5

**D**

PROKR2/actin proteins expression (A.U)

Non-CF | CF | HBE | CfBE
---|---|---|---
0.5 | 2.0 | 1.0 | 1.5
Figure 5

Non CF

CF

PROKR2

PROKR1

Basal cells
Cilia
Capillary
airway

Goblet cells
airway

control
Figure 7

A. EG-VEGF secretion (pg/ml)

B. EG-VEGF/18S/10^7

C. BV8/18S/10^11

D. BV8L/18S/10^14

E. PROKR1

F. PROKR2