Title: CFTR dysfunction induces vascular endothelial growth factor synthesis in airway epithelium

Martin C¹,²,* , Coolen N¹,²,* , Wu YZ³,⁴, Thévenot G¹, Touqui L³,⁴, Prulière-Escabasse V⁵,⁶, Papon JF⁵,⁶, Coste A⁵,⁶, Escudier E⁸,⁹, Dusser DJ¹,², Fajac I¹,⁷, Papon JF⁵,⁶, Coste A⁵,⁶, Escudier E⁸,⁹, Dusser DJ¹,², Fajac I¹,⁷, Burgel PR¹,²

Affiliations

¹ Université Paris Descartes, Sorbonne Paris Cité, Paris, France.
² Service de Pneumologie, Hôpital Cochin, AP-HP, Paris, France.
³ Unité de Défense Innée et Inflammation, Institut Pasteur, Paris, France.
⁴ INSERM U874, Paris, France
⁵ Department of Otorhinolaryngology, Hôpital Intercommunal de Créteil, Créteil, France
⁶ INSERM, U 955, Créteil, F-94000, France.
⁷ Service de Physiologie et Explorations Fonctionnelles, Hôpital Cochin, AP-HP, Paris, France.
⁸ Service de Génétique et d'Embryologie médicales, Hôpital Armand-Trousseau, AP-HP, Paris, France.
⁹ Inserm U.933, Paris, France
* These authors have contributed equally to this work.

Running head: CFTR dysfunction induces VEGF synthesis

Corresponding author: Pierre-Régis Burgel MD, PhD
27, rue du Faubourg Saint Jacques
75014 Paris, France.
Tel : + 33 1 58 41 23 67
Fax : +33 1 46 33 82 53
E-mail: pierre-regis.burgel@cch.aphp.fr
Abstract

**Background:** Peribronchial angiogenesis may occur in cystic fibrosis (CF) and vascular angiogenic growth factor (VEGF)-A regulates angiogenesis in airways.

**Methods:** Peribronchial vascularity and VEGF-A expression were examined using immunocytochemistry and morphometric analysis in lung sections obtained in 10 CF patients at transplantation vs. 10 Control non-smokers, and in two strains of C/itt-deficient mice vs. wildtype littermates. Airway epithelial NCI-H292 cells and primary cultures of non-CF human airway epithelial cells (HAEC) were treated with CFTR inhibitors (CFTR-inh\textsuperscript{172} or PPQ-102) or transfected with a CFTR siRNA with or without a selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor. Concentrations of VEGF-A and phosphorylated EGFR were measured by ELISA.

**Results:** Peribronchial vascularity was increased in CF patients, but not in C/itt-deficient mice. VEGF-A immunostaining was localized to airway epithelium and was increased in CF patients and in C/itt-deficient mice. In cultured airway epithelial cells, treatment with CFTR-inhibitors or transfection with CFTR siRNA induced a two-fold increase in VEGF-A production. CFTR inhibitors triggered epidermal growth factor receptor (EGFR) phosphorylation that was required for VEGF-A synthesis.

**Conclusions:** CF airways at transplantation showed increased peribronchial vascularity and epithelial VEGF-A expression. CFTR dysfunction triggered epithelial synthesis of VEGF-A, which may contribute to vascular remodeling.

**Keywords:** airway epithelium, cystic fibrosis transmembrane conductance regulator, vascular endothelial growth factor, epidermal growth factor receptor, angiogenesis.
**Introduction**

Cystic fibrosis (CF) is a genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (1). CFTR protein dysfunction results in abnormal ion transport across the airway epithelium (2), progressively leading to chronic lung disease (1, 3). CF lung disease is characterized by airway structural abnormalities that include bronchiectasis with mucus plugging, subepithelial fibrosis, remodeling of airway epithelium and submucosal glands, and hyperplasia of airway smooth muscle (4-6).

The bronchial circulation has the ability to proliferate and peribronchial angiogenesis (the proliferation of new blood vessels from existing vessels) is increasingly recognized as an important component in chronic airway diseases (7, 8). Abnormalities in peribronchial blood vessels may contribute to immune responses via microvascular leakage and recruitment of inflammatory cells (9, 10). Increased peribronchial vascularity was reported in asthma and in COPD (7, 8). However, to the best of our knowledge, peribronchial vascularity has not been examined in CF pathological studies.

Vascular endothelial growth factor (VEGF)-A is a key angiogenic mediator in the airways (8). Baluk et al. have shown that selective expression of VEGF-A in mouse airway epithelium triggered tracheal angiogenesis (11). Previous studies in CF subjects have reported increased VEGF-A concentrations in serum (12-14), but little is known on VEGF-A expression in CF airways. Krenn et al. found increased VEGF-A mRNA and protein in lung homogenates of CF subjects undergoing lung transplantation (15). By contrast, Meyer et al. found no increase in VEGF-A levels in bronchoalveolar lavage fluid of adult CF subjects (13). Thus, the expression pattern and the cellular localization of VEGF-A in CF airways remain to be established.
In the present study, we examined peribronchial vascularity and VEGF-A immunostaining in lung tissue sections from CF subjects vs. Control non-smokers and in the lungs of two different strains of Cftr-deficient mice vs. wildtype littermates. Because the airway epithelium appeared as the main site of VEGF-A expression in CF airways and in Cftr-deficient mice, we studied the effects of CFTR dysfunction on VEGF-A synthesis in cultured airway epithelial cells.
Methods

Human and mouse tissues

Peripheral human lung tissues (containing non-cartilaginous airways) were obtained at transplantation from 10 non-smoking CF adults and from 10 non-smoking Controls undergoing lung resection for peripheral lung cancer. Clinical characteristics of these patients are provided in an online supplement (supplementary 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 morphologic abnormalities in CF patients, tissues were obtained from two to four blocks (depending on tissue availability), whose locations were chosen randomly. In Control subjects, only one block was available for study.

*Cftr*^m1UNC* (Cftr^−/−) mice, established by gene targeting (16), and their wildtype littermates (Cftr^+/+) were obtained from the Centre de Distribution, de Typage et d’Archivage Animal (Orleans, France). *Cftr* ^m1Eur* (F508 del/del) mice (17) and their wildtype littermates (F508 wt/wt) mice were a kind gift from Pr B. Scholte (Erasmus university, Rotterdam, The Netherlands). After weaning, all mice were provided with a commercial osmotic laxative (Movicol; Norgine, Middlesex, UK) in the drinking water to prevent intestinal occlusion. Eight to ten week-old mice were sacrificed and lungs were fixed in 4% formalin and embedded in paraffin. Studies were performed on five to seven animals per group. The study conformed to the Declaration of Helsinki and all to the rules of the local Committees on Human and on Animal Research. Informed consent was obtained in all patients.
**Immunohistochemical staining and quantitative morphometric analysis**

Immunohistochemical staining was performed on 5 µm paraffin-embedded sections, as previously described (18). The following primary antibodies were used: a polyclonal Ab to VEGF-A (A 20, 1:200; Santa Cruz Biotechnology); a polyclonal Ab to the endothelial marker von Willebrand factor (vWF, 1:50; DakoCytomation) to identify blood vessels. Antigen unmasking by incubation with protease from *Streptomyces griseus* (0.01%, Sigma) for 20 min was required to improve the quality of staining for vWF. Biotinylated anti-rabbit Ab (1:200; Vector Laboratories, Burlingame, CA, USA) was used for secondary Ab, and bound Ab were visualized according to standard protocols for avidin-biotin-peroxidase complex method (Elite ABC kit; Vector Laboratories). Tissue sections were counterstained with haematoxylin. Omission of primary Ab and incubation with irrelevant immunoglobulins were used as negative controls.

Quantification of peribronchial vascularity and of VEGF-A epithelial immunostaining were performed by point counting, as previously described (4, 18, 19). Volume occupied by VEGF-A positively-stained cells in epithelium was expressed per volume of epithelium. Peribronchial vascularity was defined as the volume occupied by vWF-positive blood vessels in the peribronchial space (defined as the space between epithelial basement membrane and the base of alveolar attachments) per volume of peribronchial space. All analyses were performed on at least twenty randomly-chosen high-magnification (400X) photomicrographs obtained in two to four distant sections per patient or per mouse.

**Culture of human airway epithelial cells**

Human airway epithelial NCI-H292 cells, which express functional CFTR (20) and produce VEGF-A (18), were plated at $10^5$ cells/ml in 24-well plates (BD Falcon) and grown in
RPMI 1640 medium containing 10% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), and HEPES (25 mM) at 37°C in a humidified 5% CO2 water-jacketed incubator.

Primary cultures of human airway epithelial cells (HAEC) were obtained from five non-CF patients undergoing surgery for nasal polyps (NP), as previously described (21). Cells were plated at 10^6 cells/well in inserts (12 mm; Transwell, Costar, Cambridge, MA, USA) with 12-mm-diameter type IV collagen-coated (Sigma) polycarbonate microporous membranes and were incubated with 1 ml of DMEM/F12/antibiotics containing 2% Ultroser G (Gibco BRL, LifeTechnologies). Cells were cultured at an air/liquid interface for 3 weeks, at a time when they show full differentiation with ciliated and mucous cells (22). Previous studies have shown that these cells (A) produce VEGF-A protein with a peak of production during the first week of culture, and a decreased production thereafter until day 21 of culture (21) (B) have no CFTR expression and function during the first week of culture, but express functional CFTR after 2 weeks of culture (23). Thus, studies using CFTR inhibitors (see below) were performed at day 21 of culture.

**CFTR inhibition in human airway epithelial cells**

Inhibition of CFTR-mediated ion transport was obtained by treating airway epithelial cells with selective CFTR inhibitors (CFTR-inh^{172} or PPQ-102; Calbiochem, Nottingham, UK) (24, 25), which have been reported to stabilize the CFTR channel in a closed state. In brief, NCI-H292 cells at 60-80% confluence were treated with CFTR-inh^{172} (10^{-5} M) or with PPQ-102 (10^{-5} M) diluted in DMSO, or with DMSO alone (Sigma) for 72h. Time of exposure and concentrations of inhibitors were selected based on previous literature (24-26). In preliminary experiments, we found that CFTR inhibitors induced VEGF-A synthesis when cells were serum-starved (no FCS)
during the last 48h of culture and when cells were cultured in the presence of FCS (data not shown); all subsequent experiments were performed in FCS-containing medium. Because HAEC were cultured at the air/liquid interface, and because CFTR is expressed on the apical side of epithelial cells, treatments were applied both at the basolateral level (in culture medium), and at the apical level by dropping 150µL of culture medium on the cell surface (26). Culture media were changed daily. After 72h of incubation with CFTR inhibitors or with DMSO, cell culture media were collected and stored at -80°C. VEGF-A concentrations, corresponding to VEGF-A production in 24h, were measured by ELISA (see below).

Knockdown of CFTR expression was obtained by transfecting NCI-H292 cells with a specific CFTR small interfering (si) RNA (Sigma), and non-targeted siRNA was used as a negative control (27). Transfections were performed using TransIT-siQUEST1, according to the manufacturer’s instructions (Mirus, Madison, WI, USA) (27). As reported in a previous manuscript by our group (27), CFTR siRNA resulted in a 60 to 70% knockdown in CFTR expression, as examined by western-blotting (data not shown). Twenty-four hours after transfection, cells were rinsed 3 times with fresh culture medium and incubated for additional 24h before harvesting culture media that was stored at -80°C.

In selected experiments, epithelial cells were incubated for 24h with a selective epidermal growth factor receptor inhibitor (EGFR; AG1478, 10^{-5}M; Calbiochem) or its inactive analog (AG9, 10^{-5} M; Calbiochem) (28). For assessing the effect of CFTR inhibition on EGFR activation, NCI-H292 cells were treated with CFTR-inh^{172} or DMSO alone for 15 min, 1h, 3h, 6h, 8h and 24h. At each time point, cells were washed twice with sterile phosphate buffered saline and cell lysates were obtained by solubilizing cells at 10^{7} cells/mL in lysate buffer (1% NP-40 Alternative, 20mM Tris at pH 8.0, 137 mM NaCl, 10% glycerol, 2mM EDTA, 1mM
activated sodium orthovanadate, 10 µg/ml aprotinin, 10 µg/mL leupeptin). Samples were allowed to sit on ice for 15 min before storage at -80°C.

**Measurements of VEGF-A and phosphorylated EGFR protein concentrations**

VEGF-A in cell culture supernatants and phosphorylated EGFR (EGFR-P) in cell lysates were measured by sandwich ELISA kits according to the manufacturer’s instructions (DuoSet IC; R&D Systems; Minneapolis, MN, USA). EGFR-P results were expressed as pg of EGFR-P/µg of total protein. Protein concentrations in cell lysates were measured using a bicinchoninic acid-based protein assay kit (Pierce, Rockford, IL). Each sample was measured in duplicate.

**Quantitative PCR for VEGF mRNA**

Total RNA was extracted from NCI-H292 cells using RNeasy mini kit (Qiagen, USA). One microgram of total RNA was used for reverse transcription following quantitative PCR (qPCR) amplification of VEGF-A and β-actin expression using 7900HT fast real-time PCR system (Applied Biosystems). The qPCR primers were used as VEGF-A: 5’-AGGGCAGAATCATCACGAAGT-3’ (F), 5’-AGGGTCTCGATTGGATGGCA-3’ (R) and β-actin: 5’-GCAAACGTGCGTGACAT-3’ (F), 5’-TCGCGACAGGTCTTTGC-3’ (R). The relative quantification (RQ) of gene expression was calculated using ΔΔCt method and results were presented as fold increase compared to corresponding control at each time-point.
Statistical analysis

Data obtained from morphometric analysis were presented as % and were analyzed using the non-parametric Mann-Whitney U test and the Spearman’s rank correlation. The interobserver coefficients of variation for morphometric measurements were less than 15%. Data obtained from cell culture experiments were presented as mean ± SEM of at least 3 independent experiments performed in duplicate and were analyzed using one-way analysis of variance (ANOVA) for repeated measurements, followed by the post-hoc Student-Neuman-Keuls test for multiple comparisons. All analyses were performed using the Prism 5 software (GraphPad Inc., USA). Values of $P \leq 0.05$ were considered to indicate statistical significance.
Results
Peribronchial vascularity and VEGF-A immunostaining in human and mouse airways

In Control non-smokers, the peribronchial space was thin and immunostaining for vWF identified sparse peribronchial blood vessels. In CF subjects, the peribronchial space was markedly thickened and contained numerous blood vessels. Morphometric analysis showed that peribronchial vascularity was increased by two-fold in CF airways (Figure 1A, \( P<0.01 \)). Immunostaining for VEGF-A was positive in lung sections of all Control and CF subjects. Staining for VEGF-A was predominantly localized to the airway epithelium, where it was present in ciliated, but not in goblet cells. In CF airways, VEGF-A staining was also present in macrophages, but not in neutrophils (not shown). Morphometric analysis showed that VEGF-A staining was markedly increased in the airway epithelium of CF subjects (Figure 1B, \( P<0.05 \)).

When grouping both Controls and CF subjects, a strong correlation between epithelial VEGF-A-stained volume density and peribronchial vascular volume density was found (Figure 1C, \( r=0.65; P<0.05 \)). However, this correlation was not significant when limiting the analysis to CF subjects (\( r=0.45; P=0.19 \)).

In \( \text{Cfr}^{-/-} \) and \( \text{F508del/del} \) mice and in wildtype mice, immunostaining for VEGF-A was also localized to airway epithelium. Morphometric analysis showed increased VEGF-A-staining in the epithelium of \( \text{Cfr}^{-/-} \) and \( \text{F508del/del} \) mice compared to their respective wildtype littermates (Figure 2). No difference in peribronchial vascularity was found when comparing \( \text{Cfr}^{-/-} \)-deficient and wildtype mice (data not shown).

Representative photomicrographs of immunostaining for VEGF-A in humans and in mice are shown in Figure 3. Representative photomicrographs of vWF immunostaining in human subjects are presented in the online supplement (supplementary figure 1).
Effect of CFTR inhibition on VEGF-A synthesis in cultured human airway epithelial NCI-H292 cells

Measurement of short-circuit current confirmed that NCI-H292 cells had chloride transport that was inhibited by the selective CFTR inhibitors, CFTR-inh\textsuperscript{172} or PPQ-102 (see Online supplement, Supplementary Figure 2). Treatment of NCI-H292 cells with CFTR-inh\textsuperscript{172} (10^{-5} \text{M}) or with PPQ-102 (10^{-5} \text{M}) induced a two-fold increase in VEGF-A synthesis (Fig. 4A; \( P<0.05 \) vs. Baseline). Similarly, transfection of NCI-H292 cells with a specific CFTR siRNA increased VEGF-A synthesis (Fig. 4B; \( P<0.05 \) vs. Baseline), whereas transfection with a non-targeted siRNA had no effect.

To examine whether the effect of CFTR inhibition on VEGF-A synthesis was related to a transcriptional effect, we examined VEGF-A mRNA expression using qPCR. Treatment of NCI-H292 cells with CFTR-inh\textsuperscript{172} (10^{-5} \text{M}) induced increased VEGF-A mRNA at 12h, peaked at 24h and then decreased (see Fig. 4C).

Role of epidermal growth factor receptor (EGFR) phosphorylation in VEGF-A synthesis induced by CFTR inhibition in NCI-H292 cells

Because EGFR phosphorylation is required for VEGF-A synthesis induced by various stimuli in airway epithelial cells (18, 29), we examined its role in VEGF-A synthesis induced by CFTR inhibition. Treatment of NCI-H292 cells with CFTR inhl\textsuperscript{172} induced a significant increase in EGFR phosphorylation at 8 hours (Fig. 4D; \( P<0.05 \) vs. Vehicle, and \( P<0.05 \) vs. CFTR-inh\textsuperscript{172} at 15 min). Pretreatment of NCI-H292 cells with AG1478 (10^{-5} \text{M}, a selective inhibitor of EGFR tyrosine kinase) prevented VEGF-A synthesis induced by CFTR chemical inhibitors or by CFTR
siRNA (Fig. 4A and 4B; \( P<0.05 \) vs. Baseline). Pretreatment of NCI-H292 cells with AG9 (10\(^{-5}\) M, an inactive analog of AG1478) was without effect.

**Role of functional CFTR in VEGF-A synthesis in primary cultures of human airway epithelial cells**

Primary cultures of HAEC isolated from freshly excised nasal polyps secreted VEGF-A during the first week of culture, which gradually decreased over time, confirming previously published data (21); see Online supplement, supplementary Figure 3. At day 21 of culture, differentiated HAEC produced low levels of VEGF-A, but treatment with CFTR-inh\(^{172}\) increased VEGF-A synthesis, which was prevented by AG1478 (Fig. 4E; \( P<0.05 \)).
Discussion

We found that the airway epithelium was the main site of VEGF-A immunostaining in the lung of CF subjects obtained at transplantation, and that VEGF-A expression was increased in CF as compared with Control subjects. Immunostaining for VEGF-A was also increased in the airway epithelium in two different mouse strains with Cftr dysfunction. In cultured airway epithelial cells, inhibition of CFTR (using chemical inhibitors or siRNA) resulted in increased VEGF-A synthesis, which required EGFR activation. These data suggest that CFTR dysfunction contributes to VEGF-A upregulation in the airway epithelium of CF subjects. They further suggest roles for epithelial VEGF-A in the peribronchial vascular remodeling that occurs in CF airways.

We report for the first time increased expression of VEGF-A in CF airway epithelium. McColley et al. reported increased serum VEGF-A levels in stable CF subjects vs. subjects with non-CF pulmonary diseases (12), but VEGF-A levels were not measured in the airway compartment. Meyer et al. measured similar concentrations of VEGF-A in the bronchoalveolar lavage fluid (BALF) of 9 CF and 16 age-matched healthy volunteers (13). Because VEGF-A was degraded in vitro by CF BALF, which contained high neutrophil elastase activity, these authors were unable to derive definitive conclusion on pulmonary production of VEGF-A in CF subjects (13). Krenn et al. found increased VEGF mRNA and protein in lung homogenates of CF subjects at transplantation as compared with lungs obtained in non-smokers (15). Our data confirm these latter findings and extend these data by showing that the airway epithelium is the main site of VEGF-A expression in CF lungs.

Our conclusion that CFTR dysfunction triggers VEGF-A synthesis is based on data obtained in cultured airway epithelial cells. Previously, Verhaeghe et al. reported increased
VEGF-A synthesis in a human fetal cell line carrying the homozygous mutation F508del, as compared to a control human fetal cell line (30). These latter data provided circumstantial evidence of a role for CFTR dysfunction in VEGF-A production; however differences between these two cell lines could have been unrelated to CFTR dysfunction (31). In the present study, inhibition of CFTR channel function using chemical inhibitors, or knockdown of CFTR using CFTR siRNA in a non-CF cell line and in non-CF HAEC provided direct evidence that CFTR dysfunction triggered VEGF-A synthesis. Increased immunostaining for VEGF-A in the airway epithelium of Cftr-deficient mice provided further evidence for this finding.

The finding that increased VEGF-A expression was not associated with increased peribronchial vascularity in Cftr-deficient mice was somewhat surprising. Because VEGF-A stimulates endothelial cell proliferation and angiogenesis via its effects on VEGFR2 (32), we examined the expression of VEGFR2 in mouse lung. In wildtype and in Cftr-deficient (both Cftr -/- and F508del/del) mice, immunostaining for VEGFR2 was localized in the airway epithelium, but was absent in the endothelium of peribronchial blood vessels (see Online supplement, supplementary Figure 4). By contrast, Baluk et al. found that VEGF-A overexpression in airway epithelium, which resulted with tracheal angiogenesis, was associated with expression of VEGFR2 in the endothelium of tracheal blood vessels (11). We suggest that the lack of VEGFR2 expression in endothelium may have accounted for the absence of increased vascularity in Cftr-deficient mice.

The strength of our study relies in the use of complementary models and sources of information, including human CF tissues, mouse models and cultured human airway epithelial cells. We also recognize limitations to our approaches. The correlation that was found between VEGF-A in epithelium and peribronchial vascularity was significant only when grouping
Controls and CF subjects. The lack of correlation between peribronchial vascularity and epithelial VEGF-A in CF subjects may be related to the relatively small number of CF tissues available, and to the homogeneous characteristics of these patients who were recruited at transplantation. Alternatively, epithelial VEGF-A may not be associated with peribronchial angiogenesis in CF subjects and play different roles (e.g., epithelial proliferation (33)). CF subjects at transplantation were younger than Controls. Because a previous study of VEGF-A immunostaining in young adults with normal lung function reported no immunostaining in the airway epithelium (34), this difference was unlikely to affect our findings. Mouse models of CFTR dysfunction do not reproduce the structural abnormalities typical of human CF lung disease (35). Thus, the absence of increased peribronchial vascularity in Cfr-deficient mice should be interpreted cautiously. However, one might hypothesize that beside CFTR dysfunction, other factors inducing VEGF-A expression, such as bacterial infection (18), might be necessary to promote peribronchial angiogenesis. Finally, CFTR inhibition induced both increased VEGF-A mRNA and protein synthesis, suggesting a transcriptional effect. However, further studies will be necessary to determine whether the effect is related to mRNA synthesis or mRNA stabilization.

Our findings indicated that CFTR dysfunction triggered VEGF-A synthesis in airway epithelium, independently of infection and inflammation. However, increased VEGF-A expression that was present in CF airway epithelium at transplantation could have been related to multiple factors: proinflammatory cytokines (e.g. TNF-\( \alpha \), IL-1\( \beta \)), microbial products from Gram negative and Gram positive bacteria (18, 29) and viruses (29), and hypoxia (36) have also been reported to increase VEGF-A synthesis in airway epithelium.

VEGF-A was localized to ciliated cells, probably as a result of EGFR activation in ciliated cells or in precursors of ciliated cells (e.g., basal cells). Other major products of EGFR
activation in the airway epithelium (e.g., mucins and IL-8) have been localized in secretory cells (4). The localization of EGFR in airway epithelial cell subpopulation is somewhat controversial in the literature: Tyner et al. reported selective expression of EGFR in ciliated cells in vivo and in cultured epithelial cells, and suggested transdifferentiation of ciliated cells into mucin producing cells (37). Others have found EGFR expression in basal cells and in non-granulated secretory cells, but not in ciliated cells (4, 38). It is suggested that EGFR localization is dynamic process that may vary under various pathophysiological conditions.

Inhibition of CFTR function using CFTR-inh\textsuperscript{172} triggered EGFR phosphorylation, and increased VEGF synthesis after CFTR inhibition required EGFR activation in airway epithelial cells. Treatment of NCI-H292 cells with CFTR-inh\textsuperscript{172} resulted in the inhibition of chloride transport within minutes, confirming previous findings by Perez et al. (26), but EGFR phosphorylation was increased at 8h. Because activation of EGFR by its ligands (e.g., EGF or TGF-alpha) usually occurs within seconds, this data indicates that CFTR-inh\textsuperscript{172} is not an EGFR ligand and suggests that activation of EGFR under these circumstances occurs via a cascade of events in epithelial cells. The precise molecular mechanisms leading to EGFR activation remain to be established.

Increased VEGF-A synthesis in airway epithelium could contribute to angiogenesis (11, 30) and increase in vascular permeability, facilitating recruitment of inflammatory cells (e.g., neutrophils) and proteins in the airways. Further, peribronchial angiogenesis could contribute to the occurrence of massive hemoptysis, a potentially life-threatening complication in CF patients (39). Massive hemoptysis in CF patients is usually treated by bronchial artery embolization. High rates of recurrence are observed after embolization in these patients, presumably related to ongoing peribronchial angiogenesis and inflammation. Recurrent hemoptysis uncontrolled by
repeated embolization is a recognized criterion for lung transplantation in CF patients. We suggest that therapies directly targeting angiogenesis may be useful in CF patients with recurrent hemoptysis, but further studies will be necessary before such therapies may be considered in humans.

In summary, VEGF-A expression was markedly increased in CF airway epithelium at transplantation, suggesting roles in peribronchial angiogenesis in CF patients (11). CFTR dysfunction directly contributed to VEGF-A upregulation in airway epithelium via EGFR activation. Therapies directly targeting CFTR dysfunction (40, 41) may result in significant reduction of VEGF-A synthesis and of peribronchial vascularity. Additionally, growth factor (e.g., EGF and/or VEGF) receptors antagonists could represent a promising way for reducing vascular remodeling in CF airways.

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Figure legends

Figure 1: Morphometric analysis of peribronchial vascularity and VEGF-A immunostaining in the airways of Controls (n=10) and of CF subjects (n=10).

Airway sections were immunostained for the endothelial marker vWF (to identify blood vessels) or for VEGF-A (VEGF). Morphometric analysis was performed using point counting (see Methods). A. Peribronchial vascularity was defined as the volume occupied by blood vessels (vascular volume) in the peribronchial space/volume of peribronchial space (%). B. VEGF-A positively-stained volume in epithelium. C. Correlation between VEGF-A in epithelium and peribronchial vascularity was performed using the non-parametric Spearman’s rank correlation. This correlation was highly significant (r=0.65; P<0.05) when considering both Control and CF subjects, but was not significant when considering only CF subjects (r=0.45; P=0.19). Each square correspond to results obtained in a Control subject (open square) or in a CF patients (solid square). Horizontal bars represent medians.
Figure 2: Morphometric analysis of VEGF-A immunostaining in the airway epithelium of *Cftr*-deficient and wildtype mice.

Airway sections were obtained in *Cftr*<sup>m1UNC</sup> (*Cftr<sup>−/+</sup>*) mice and their wildtype (*Cftr<sup>+/+</sup>*) littermates (left), and in *Cftr*<sup>tm1Eur</sup> (*F508<sup>del/del</sup>*) and their wildtype (*F508<sup>wt/wt</sup>*) littermates (right). Sections were immunostained for VEGF-A and morphometric analysis was performed as described in Methods. Each triangle corresponds to results obtained in a *Cftr*-wildtype (*open triangle*) or in a *Cftr*-deficient (*solid triangle*) mouse. Horizontal bars represent medians.
Figure 3: Representative photomicrographs of VEGF-A immunostaining in human and in mouse airways.

Airway sections were obtained in human Control patients versus Cystic Fibrosis patients (upper panels), in Cftr +/+ versus Cftr -/- mice (middle panels) and in F508 wt/wt versus F508 del/del mice (lower panels). Sections were stained with an Ab to VEGF-A (brown color) and counterstained with haematoxylin. In Control patients and in Cftr +/+ and F508 wt/wt mice, only sparse staining for VEGF-A was present in the epithelium. In CF patients, and in both Cftr -/- and F508 del/del mice, extensive staining for VEGF-A was present in epithelium. Photomicrographs were representative of results obtained in 10 Controls and in 10 CF patients, in 7 Cftr +/+ and Cftr -/- mice, and in 5 F508 wt/wt and F508 del/del mice. Scale bars = 50µm. Original magnification: 200 X.
Figure 4: Effect of CFTR inhibition on VEGF-A synthesis and EGFR activation in airway epithelial cells.

A: Effect of selective CFTR inhibitors on VEGF-A synthesis in NCI-H292 cells: Cells were cultured in culture medium alone (Baseline; open bar), containing DMSO (Vehicle; grey bar) or
containing selective CFTR inhibitors (CFTR-inh\textsuperscript{172} or PPQ-102; 10\textsuperscript{-5}M; solid bars). VEGF-A concentrations were measured by ELISA (see Methods). In some experiments, cells were pretreated with the selective EGFR tyrosine kinase inhibitor AG1478 (10\textsuperscript{-5}M) or with its inactive analog AG9 (10\textsuperscript{-5}M). Results were expressed as mean ± SEM of n ≥ 3 independent experiments in duplicate. *, P<0.05 vs. Baseline; †, P<0.05 vs. CFTR-inh\textsuperscript{172} alone; and ††, P<0.05 vs. PPQ-102 alone.

**B: Effect of transfection with a CFTR siRNA on VEGF-A synthesis in NCI-H292 cells.** Cells were cultured in cell culture medium alone (Baseline; open bar), or transfected with a non-targeted (scrambled) siRNA (grey bar) or with a specific CFTR siRNA (solid bars; see Methods). Results were expressed as mean ± SEM, n ≥ 3 independent experiments in duplicate. *, P<0.05 vs. Baseline; †, P<0.05 vs. CFTR siRNA alone.

**C. Effect of CFTR-inh\textsuperscript{172} on VEGF-A mRNA expression in NCI-H292 cells.** Cells were cultured in culture medium alone (Baseline, open bar), or containing CFTR-inh\textsuperscript{172} (10\textsuperscript{-5}M; solid bars). VEGF mRNA was quantified using qPCR and reported as relative quantity (RQ). n=3 independent experiments in duplicates. *, P<0.05 compared to Baseline at each time point.

**D: Effect of CFTR-inh\textsuperscript{172} on EGFR phosphorylation in NCI-H292 cells:** NCI-H292 cells were cultured in culture medium containing DMSO (grey bars) or containing CFTR-inh\textsuperscript{172} (10\textsuperscript{-5}M; solid bars). Cell lysates were collected at different time points, and phosphorylated EGFR (EGFR-P) concentrations were measured by ELISA (see Methods). Results were expressed as mean ± SEM from n ≥ 3 independent experiments in duplicate. *, P<0.05 versus DMSO at 8h and **, P<0.01 versus CFTR-inh\textsuperscript{172} at 15 min.

**E. Effect of CFTR-inh\textsuperscript{172} on VEGF-A synthesis in primary culture of HAEC at the air/liquid interface:** At day 21 of culture, HAEC were cultured in culture medium alone
(Baseline; *open bar*) or containing CFTR-inh$^{172}$ ($10^{-5}$M; solid bars) for 72 hours. Results were expressed as mean ± SEM from n=5 independent experiments in duplicate. *, $P<0.05$ vs. Baseline; †, $P<0.05$ vs. CFTR-inh$^{172}$ alone.