ONLINE SUPPLEMENT

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

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Supplementary Methods

Patients

Clinical characteristics of Control and cystic fibrosis (CF) patients included in the study are presented in **supplementary table 1.**

Supplementary table 1. **Clinical characteristics of Control and CF subjects.**

	Controls (n=10)	**CF (n=10)**
Male sex, n	3	3
Age, years	66 (10)	36 (6)
FEV_1, % predicted	97 (20)	22 (7)
FVC, % predicted	101 (23)	33 (9)
***CFTR* genotype**, n		
F508del / F508del	-	7
F508del /N1303K	-	1
F508del /Q890X	-	1
G542X/3849	-	1
Bronchial colonization *, n		
P. aeruginosa	0	10
S. aureus	0	1
H. influenzae	0	1
S. maltophilia	0	1

All data are mean (SD) or n. *Colonization with multiple pathogens is possible. FEV1: forced expiratory volume in 1 sec. FVC: forced vital capacity. CFTR: cystic fibrosis transmembrane conductance regulator.

Electrophysiological studies

Measurements of short-circuit current (I_{SC}), was performed in NCI-H292 cells treated with DMSO alone (vehicle, 2µL/mL), or with CFTR-inh^{172} or PPQ-102 (10^{-5} M) as previously described [1]. Snapwell inserts were mounted in vertical diffusion chambers and were bathed with Ringer solution (pH 7.4) continuously bubbled with 5% CO_2, 95% air at 37°C. The apical and basolateral chambers were filled with 137 mM NaCl, 5.6 mM KCl, 1.9 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.9 mM CH_3COONa, 1.3 mM NaH_2PO_4, 10 mM HEPES, and 10 mM glucose. Potential difference was short circuited to 0 mV with a voltage clamp (World Precision Instruments, Astonbury, UK) connected to the apical and basolateral chambers *via*

Ag-AgCl electrodes and agar bridges to measure I_{SC}. I_{SC} was allowed to stabilize before adding the drugs. Amiloride (10^{-4} M) was applied to the apical solution to calculate the amiloride sensitive part of I_{sc} ($\Delta I_{sc\ amil}$), which is the difference between I_{sc} measured in the absence and presence of amiloride. Amiloride treated NCI-H292 cells were then stimulated with forskolin (10^{-5} M, basolateral side) and IBMX (10^{4} M, basolateral side) to induce cAMP-dependent Cl^- secretion ($I_{sc\ IBMX+forsk}$). $\Delta I_{sc\ IBMX+forsk}$ was the difference between the initial value of I_{sc} and the peak value obtained in response to drug addition.

Time course of VEGF-A synthesis in primary culture of human airway epithelial cells at air/liquid interface

Cell culture media were collected in baseline condition at different time-points (day 4, 7, 14 and 21) after cell plating for VEGF-A concentration measurements by ELISA (pg/mL). The results were expressed by mean ± SEM from n = 3 primary cultures from 3 different non-CF patients, realized in triplicate conditions.

Supplementary Results

Peribronchial vascularity in human 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. Representative photomicrographs of peribronchial vascularity in Controls and in CF subjects at transplantation are presented in **supplementary Figure 1.**

Effect of CFTR inhibitors on short-circuit current measurement in NCI-H292 cells

A c-AMP-dependent current was observed in NCI-H292 cells under basal conditions (mean±SD ΔI_{sc} 2.32±0.79 μA/cm^2) and with Vehicle (ΔI_{sc} 2.28±0.60 μA/cm^2). (See **supplementary Figure 2).** As CFTR-inh^{172} or PPQ-102 treatment totally inhibited the c-AMP-dependent current, we confirmed that NCI-H292 cells expressed a functional chloride CFTR channel.

Time course of VEGF-A production in HAEC under baseline condition (day 4-21).

VEGF-A concentration in HAEC culture medium was maximal at day four and gradually decreased over time (**supplementary Figure 3**).

Immunostaining for VEGFR2 in mouse lungs (supplementary Figure 4)

VEGFR2 was not expressed in endothelium of peribronchial blood vessels but was expressed in the airway epithelium.

Supplementary figure legends

Supplementary Figure 1: Representative photomicrographs of peribronchial vascularity in human airways. Airway sections were obtained in human Control non-smokers (left) *versus* Cystic fibrosis patients at transplantation (right). Sections were stained with an Ab to vWF (brown color) used as an endothelial marker and counterstained with haematoxylin. In Control non-smokers, the peribronchial space was thin and immunostaining for vWF identified sparse peribronchial blood vessels (arrowheads). In CF subjects, the peribronchial space was markedly thickened and contained numerous blood vessels. Photomicrographs are representative of morphological quantification results obtained in 10 Control non-smokers and in 10 CF subjects. Scale bars = 100 µm. Original magnification: 100 X.

Supplementary Figure 2: Effect of CFTR inhibitors on short-circuit measurement in NCI-H292 cells. When NCI-H292 cells were exposed to CFTR inhibitors (CFTR-inh^{172} and PPQ-102), no changes in short-circuit current after forskolin and IBMX stimulation in amiloride pre-treated cells was observed, whereas in baseline cells and cells exposed only to DMSO (vehicle), amiloride-pretreated cells responded to forskolin and IBMX stimulation. Results are expressed as mean ± SEM of n = 3 independent experiments in duplicate. *, $P<0.05$ *vs.* Baseline; **, $P<0.05$ *vs.* Vehicle.

Supplementary Figure 3: Time course of VEGF-A production in HAEC under baseline condition (day 4-21). Cell culture media were collected in baseline condition at different time-points (day 4, 7, 14 and 21) after cell plating for VEGF-A concentration measurements by ELISA (pg/mL). The results are expressed by mean ± SEM from n = 3 primary cultures from 3 different non-CF patients, realized in triplicate conditions.

Supplementary Figure 4: Representative photomicrographs of VEGFR2, Flk-1 immunohistochemical staining in Cftr-deficient mice airways.

Airway sections were obtained in Cftr-deficient mice (left: *Cftr* -/-; right: *F508del/del*). Sections were stained with a rabbit polyclonal Ab to VEGFR2, Flk-1 (Santacruz biotech; brown color) and counterstained with haematoxylin. In both mice strains, immunohistochemical staining for VEGFR2, Flk1 was found in the airway epithelium (brown color), but not in the endothelium of peribronchial blood vessels (arrows). Scale bars = 200 µm. Original magnification: 100 X.

References

1. Prulière-Escabasse V, Fanen P, Dazy AC, Lechapt-Zalcman E, Rideau D, Edelman A, et al. TGF-beta 1 downregulates CFTR expression and function in nasal polyps of non-CF patients. Am J Physiol Lung Cell Mol Physiol. 2005;**288**:L77-L83.