RAPID REPORT

The Pathophysiology of COVID-19 and SARS-CoV-2 Infection

Angiotensin-converting enzyme 2 expression in COPD and IPF fibroblasts: the forgotten cell in COVID-19

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Abstract

The COVID-19 pandemic is associated with severe pneumonia and acute respiratory distress syndrome leading to death in susceptible individuals. For those who recover, post-COVID-19 complications may include development of pulmonary fibrosis. Factors contributing to disease severity or development of complications are not known. Using computational analysis with experimental data, we report that idiopathic pulmonary fibrosis (IPF)- and chronic obstructive pulmonary disease (COPD)-derived lung fibroblasts express higher levels of angiotensin-converting enzyme 2 (ACE2), the receptor for SARS-CoV-2 entry and part of the renin-angiotensin system that is antifibrotic and anti-inflammatory. In preclinical models, we found that chronic exposure to cigarette smoke, a risk factor for both COPD and IPF and potentially for SARS-CoV-2 infection, significantly increased pulmonary ACE2 protein expression. Further studies are needed to understand the functional implications of ACE2 on lung fibroblasts, a cell type that thus far has received relatively little attention in the context of COVID-19.

ACE2; COPD; fibroblast; IPF; SARS-CoV-2

INTRODUCTION

In December 2019, a newly identified novel coronavirus called SARS-CoV-2 spread rapidly through the world, causing an outbreak of coronavirus disease-2019 (COVID-19) (1). COVID-19 is associated with a myriad of symptoms ranging from asymptomatic to severe pneumonia and acute respiratory distress syndrome leading to death (2). There are at least 20 million COVID-19 cases worldwide and hundreds of thousands of deaths. SARS-CoV-2 infects humans by binding to the human angiotensin-converting enzyme 2 (ACE2). Pulmonary ACE2 is concentrated in type II alveolar cells and bronchial and tracheal epithelial cells, which serve as an entry point for viral infection (3). Changes in the expression of ACE2 may contribute to susceptibility of SARS-CoV-2. Environmental factors such as smoking are linked to an increase in ACE2 expression. Although controversial, high ACE2 expression may explain the risk of severe COVID-19 in certain populations, including those with underlying health conditions linked to smoking, such as chronic obstructive pulmonary disease (COPD) (4, 5). Even among patients with COVID-19 who recover, there is the potential for post-COVID-19 complications, particularly the development of pulmonary fibrosis (6, 7).

Although most studies have focused on epithelial expression, ACE2 in other cell types within the lung may also be important. Fibroblasts have been shown to harbor viral particles from SARS-CoV-2 in patients with COVID-19 (8). It is well accepted that fibroblasts provide structure to the lungs and other organs by synthesizing extracellular matrix (ECM) proteins and are increasingly recognized to play key roles in innate immunity (9). In pulmonary fibrosis, fibroblasts are the key cell type involved in excessive ECM deposition that stiffens the lungs. In COPD, loss of lung fibroblast repair function may contribute to the emphysema component of this disease and cause small airway fibrosis that limits airflow (10). When we also consider that ACE2 is part of the renin-angiotensin-system (RAS) branch that is antifibrotic and anti-inflammatory (11), knowledge regarding the expression of ACE2 in lung fibroblasts is highly relevant. Here, we sought to determine the expression of ACE on COPD-
and IPF-derived lung fibroblasts and provide direct evidence on whether cigarette smoke changes pulmonary ACE-2 levels.

MATERIALS AND METHODS

Computational analysis of single-cell ACE2 expression. The single-cell data used for the analyses were downloaded from https://advances.sciencemag.org/content/6/28/eaba1983, which profiled 32 IPF lungs, 18 COPD lungs, and 28 control donor lungs (12). The data set was further processed using the scopy (13) and scdiff (14) software. Cells expressing fewer than 200 genes or with more than 40% of mitochondrial reads were filtered. The expression was transformed into the log space (log1p). After the processing, we got 312,922 cells in total, including 147,167 IPF cells, 69,452 COPD cells, and 96,303 control cells.

Derivation and culture of lung fibroblasts. For COPD, fibroblasts were derived from lung tissues of subjects undergoing lung resection surgery at McMaster University as previously described (15). Patient characteristics are mentioned in Ref. (15), and a subset of these cells were used in this study that included never-smokers [control; male (M)/female (F) = 1/3; age, 71 ± 7 yr] as well as current smokers with (COPD; M/F = 2/2; age, 61 ± 5.4 yr) and without COPD (smoker; M/F = 1/3; age, 61.3 ± 4.8 yr). For IPF fibroblasts, control (M/F = 2/2; age, 35 ± 11.05 yr) and IPF-derived [3 M (1 unknown); age, 58.7 ± 6.8 yr] primary normal lung fibroblasts were cultured from the outgrowths of histologically normal

Fig. 1. Cell-specific ACE2 expression in COPD and IPF lungs. A: expression of ACE2: data analysis of scRNA-seq of COPD, IPF, and control lungs revealed that there were more ACE2+ cells in COPD. B: percentage (%) of ACE2+ cells: ACE2+ cells accounted for 2.94% of the IPF cells, 2.40% of the COPD cells, and 2.30% of control cells. C: cell-specific ACE2+ percentage (%): ACE2+ cells are mostly enriched in lung epithelial cells (goblet cells, 6.6%; basal cells, 5.6%). Fibroblasts (4.0%) are also enriched with ACE2, ranking 9th of all 38 cell types. Results were analyzed with a one-sided Mann–Whitney U test. ACE2, angiotensin-converting enzyme 2; COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis.
regions of lungs that were provided for organ donation but were later deemed unsuitable for transplantation (Gift of Life, Michigan). Cells were isolated and cultured as previously described (16).

**Western blot.**
Total cellular protein was extracted using RIPA lysis buffer (Thermo Fisher Scientific, Rockford) and Protease Inhibitor Cocktail (PIC, Roche). Ten to twenty micrograms of protein lysate were subjected to 10% SDS-PAGE gels and transferred onto Immun-Blot PVDF membranes (Bio-Rad Laboratories, Hercules, CA). Then, the membrane was blocked for 1 h at room temperature in blocking solution (5% w/v of nonfat dry milk in 1x PBS/0.1% Tween-20). Antibodies against ACE2 (SN0754; 1:500-1:1,000; Invitrogen, CA), actin (1:10,000; Millipore, MA), and tubulin (1:50,000; Sigma, CA) and the secondary antibodies goat anti-rabbit IgG horseradish peroxidase (HRP)-linked, (1:10,000; Cell Signaling Technologies, CA) and HRP-conjugated horse anti-mouse IgG (1:10,000, Cell Signaling) were used. Protein bands were visualized using a ChemiDoc MP Imaging System (Bio-Rad). Densitometric analysis was performed using Image Lab Software Version 5 (Bio-Rad). Protein expression was normalized to tubulin or actin, and the data presented as the fold-change. All antibodies detected the target protein at the predicted molecular weight. As an additional control, ACE2 siRNA was used to reduce ACE2 protein levels (data not shown).

**Preparation of cigarette smoke extract.**
Research-grade cigarettes (3R4F) with a filter were obtained from the Kentucky Tobacco Research Council (Lexington, KT), and cigarette smoke extract (CSE) was generated as previously described (17–20). Briefly, smoke from one cigarette was bubbled into 10 mL of serum-free media and subsequently passed through a 0.45-μm sterile filter (25-mm Acrodisc; Pall Corp., Ann Arbor, MI). An optical density of 0.65 (320 nm) was considered to represent 100% CSE. This CSE preparation was diluted to 2% in serum-free media.

**Preclinical cigarette smoke exposure.**
Mice (Jackson Laboratory; C57/BL6; age, 8–12 wk; male mice) were exposed to cigarette smoke using a whole body exposure system as we have described (InExpose; SCIREQ Inc., Montreal, Canada) (21–23). Briefly, mice were exposed to research-grade cigarettes (3R4F; University of Kentucky, Lexington, KY) for two 1-h smoke exposures per day for 3 days or for 5 days/wk for 2 or 4 wk. All animal procedures were approved by the McGill University Animal Care Committee (Protocol No. 5933) and were carried out in accordance with the Canadian Council on Animal Care. Lung tissue was collected for protein/Western blot analysis as described earlier.

**Statistical analysis.**
Statistical analysis was performed using Prism 6-1 (La Jolla, CA). Statistical differences between group mean values were determined by ANOVA followed by a Newman–Keuls multiple-comparisons test. A t test was used to determine significance between two groups. P value of <0.05 was considered significant.

## RESULTS
To first address the extent to which ACE2 gene expression differed between COPD and IPF in pulmonary cell populations, we used single-cell data profiled from 32 idiopathic

![Fig. 2](https://example.com/figure2)

**Fig. 2.** ACE2 protein is higher in COPD- and IPF-derived lung fibroblasts. A and B: COPD fibroblasts: lung fibroblasts derived from subjects with COPD had significantly higher ACE2 protein expression compared with lung fibroblasts from never-smokers (*P < 0.05). There was a trend toward higher ACE2 in smoker-derived fibroblasts. Smoker and COPD-derived lung fibroblasts were from current smokers. Data were analyzed by a one-way ANOVA followed by Dunn’s test. C and D: IPF fibroblasts: lung fibroblasts derived from subjects with IPF had significantly higher ACE2 protein compared with control cells. Data were analyzed by a two-tailed t test. Results are expressed as the means ± SE (**P < 0.001 compared with control cells) n = 4/group. ACE2, angiotensin-converting enzyme 2; COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis.
ACE2 IN LUNG FIBROBLASTS

Cigarette smoke remains a prevalent environmental toxicant and leading risk factor for both COPD and pulmonary fibrosis. This led us to wonder if exposure to cigarette smoke could directly increase ACE2 protein expression, as suggested by recent data from smoker lungs (24). First, we evaluated whether ACE2 was affected by exposure of lung fibroblasts to CSE, an in vitro surrogate for cigarette smoke (25). For these studies, lung fibroblasts from a never-smoker were exposed to 2% CSE for up to 48 h; this concentration of CSE induces an overall inflammatory and oxidative stress response in lung structural cells (18, 19). However, there was no change in total ACE2 expression upon exposure to CSE (Fig. 2A and B). There was a trend toward increased ACE2 in smoker-derived cells, but this did not reach statistical significance. In IPF lung fibroblasts, protein levels of ACE2 were also significantly increased compared with control lung fibroblasts (Fig. 2C and D).

Chronic cigarette smoke exposure increases pulmonary ACE2 protein expression. A: in vitro CSE exposure: primary human lung fibroblasts from a never-smoker were exposed to 2% CSE for 8-48 h and ACE2 protein evaluated by Western blot; normalization was done to tubulin. There was no change in total ACE2 expression upon exposure to CSE. B and C: acute exposure: mice were exposed to cigarette smoke using the SCIREQ inExpose whole body exposure system twice per day for 3 days. Control mice were exposed to room air. Mice were euthanized and the lungs were processed for Western blot analysis. There was no change in the total levels of ACE2 in the cigarette smoke-exposed mice compared with the control mouse. Number of mice in each group is indicated. Data were analyzed by a two-tailed t test. ACE2, angiotensin-converting enzyme 2; CSE, cigarette smoke extract.
no difference in ACE2 protein in response to CSE (Fig. 3A). We next used our preclinical model of cigarette smoke exposure to mimic acute and chronic exposure scenarios (21, 22). In lungs from mice exposed to a 3-day (acute) cigarette smoke, ACE2 protein expression was not significantly altered (Fig. 3, B and C). However, ACE2 protein expression was significantly increased in lungs from mice chronically exposed to cigarette smoke (Fig. 3, D and E). ACE2 protein significantly increased in those exposed to cigarette smoke for 4 wk compared with in-air-exposed controls as well as those exposed to cigarette smoke for 2 wk (normalized to actin). Thus, chronic exposure to cigarette smoke is necessary to increase ACE2 expression in the lungs.

**DISCUSSION**

COVID-19 is a pandemic disease, and people with preexisting conditions may be at a higher risk. Potential post-COVID-19 complications include the development of pulmonary fibrosis, which may be an emerging health threat for the millions who recover. Although epithelial cells are considered the primary cells for SARS-CoV-2 infection, lung fibroblasts also express ACE2 and may be an important cell type responsible for the persistence of infection, disease severity, and recovery from COVID-19. Lung fibroblasts respond directly to not only inciting agents like cigarette smoke but also cues provided by adjacent cells, including leukocytes and epithelial cells. In the context of SARS-CoV-2 infection, their release of profibrotic/inflammatory mediators such as transforming growth factor-β (TGF-β) could drive myofibroblast differentiation and consequent ECM deposition. Characterization of ACE2 in lung fibroblasts is also particularly relevant, as activation of the ACE2-Ang-(1-7)-Mas axis protects against fibrosis (26) and smoke-induced lung inflammation (27).

Our findings are also the first direct evidence that chronic (but not acute) cigarette smoke exposure increases ACE2. Although a limitation of this study is that we did not determine the specific cell type(s) in which ACE2 expression increased after smoke exposure, we would anticipate increased levels in multiple cell types, including epithelial cells and fibroblasts. Part of the reason for this is that our data also show there is an increase in ACE2 expression in COPD- and IPF-derived lung fibroblasts, a finding that may initially seem contradictory given the protective role of ACE2. It is possible that this increase reflects a compensatory response to the disease phenotype and/or the inciting agent (i.e., smoke). Whether ACE2 in fibroblasts reduces features of inflammation (i.e., cytokine expression) and fibrosis (e.g., ECM protein expression) in the context of COVID-19 is not known. It could also be that there is dysregulation of other components of the ACE2-Ang-(1-7)-Mas axis not examined in this study, particularly Mas receptor, which is downregulated by TGF-β in fibroblasts (28). One might speculate that although ACE2 could have antifibrotic properties, lower levels of the Mas receptor may abrogate these protective effects, with increased ACE2 augmenting not only susceptibility to SARS-CoV-2 infection but also post-COVID complications.

There were some additional limitations of this study, including the evaluation of a relatively small number of lung fibroblasts and that the cross-sectional nature of these data does not allow us to show causation. Nevertheless, to our knowledge, our work is the first to evaluate ACE2 expression in human lung fibroblasts, a cell type that has been overlooked in COVID-19 but whose dysfunction may contribute significantly to disease burden. More research is needed to understand the functional significance of higher ACE2 in lung fibroblasts and the potential consequence toward COVID-19 and its clinical outcomes.

**GRANTS**

This work was supported by the Canada Foundation for Innovation and the Canadian Institutes for Health Research. C. J. Bagliole was supported by a salary award from the Fonds de recherche du Quebec-Sante. N. Aloui was supported by a scholarship from Taibah University, Saudi Arabia.

**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**

S.N.A.H. and C.J.B. conceived and designed research; N.A. and H.T. performed experiments; N.A., H.T., and J.D. analyzed data; H.T., J.D., G.J.F., S.K.H., D.H.E., and C.J.B. interpreted results of experiments; J.D. and C.J.B. prepared figures; N.A. and C.J.B. drafted manuscript; N.A., H.T., G.J.F., P.N., S.K.H., D.H.E., and C.J.B. edited and revised manuscript; N.A., H.T., J.D., G.J.F., P.N., S.K.H., S.N.A.H., D.H.E., and C.J.B. approved final version of manuscript.

**REFERENCES**

1. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL, Chen HD, Chen J, Luo Y, Guo H, Jiang RD, Liu MQ, Chen Y, Shen XR, Wang X, Zheng XS, Zhao K, Chen QJ, Deng F, Liu LL, Yan B, Zhan FX, Wang YY, Xiao GF, Shi ZL. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature 579: 270–273, 2020. doi:10.1038/s41586-020-2012-7.
2. Kai H, Kai M. Interactions of coronaviruses with ACE2, angiotensin II, and RAS inhibitors-lessons from available evidence and insights into COVID-19. Hypertens Res 43: 648–654, 2020. doi:10.1038/s41440-020-0455-8.
3. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J Pathol 203: 631–637, 2004. doi:10.1002/path.1570.
4. Vardavas CI, Nikitara K. COVID-19 and smoking: a systematic review of the evidence. Tob Indus Dis 18: 20, 2020. doi:10.18332/tid/19324.
5. D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA 323: 1061, 2020., doi:10.1001/jama.2020.1585.
6. Dalan R, Bornstein SR, El-Armouche A, Rodinov RN, Markov A, Wielockz B, Beuschlein F, Boehm BO. The ACE-2 in COVID-19: foe or friend? Horm Metab Res 52: 257–263, 2020. doi:10.1055/a-1155-0501.
7. Grillo F, Barisone E, Ball L, Mastracci L, Fiocca R. Lung fibrosis: an undervalued finding in COVID-19 pathological series. Lancet Infect Dis, 2020.
8. Facchetti F, Bugatti M, Drera E, Tripodo C, Sartori E, Cancila V, Papaccio M, Castellani R, Casola S, Boniotti MB, Cavadini P, Lavazza A. SARS-CoV2 vertical transmission with adverse effects on the newborn revealed through integrated immunohistochemical, electron
ACE2 IN LUNG FIBROBLASTS

microscopy and molecular analyses of placenta. EBioMedicine 59: 102951, 2020. doi:10.1016/j.ebiom.2020.102951.

9. Krausgruber T, Fortelny N, Fife-Gerned V, Senekowitsch M, Schuster LC, Lercher A, Nemc A, Schmid C, Rendeiro AF, Berghalter A, Bock C. Structural cells are key regulators of organ-specific immune responses. Nature 583: 296–302, 2020. doi:10.1038/s41586-020-2424-4.

10. Togo S, Holz O, Liu X, Sugiura H, Kami K, Wang X, Kawasaki S, Ahn Y, Fredriksson K, Skold CM, Mueller KC, Banscheid D, Welker L, Watz H, Magnussen H, Rennard SI. Lung fibroblast functions in patients with chronic obstructive pulmonary disease are altered by multiple mechanisms. Am J Respir Crit Care Med 178: 248–260, 2008. doi:10.1164/rccm.200706-929OC.

11. Lumbers ER, Delforce SJ, Pringle KG, Smith GR. The lung, the heart, the novel coronavirus, and the renin-angiotensin system; the need for clinical trials. Front Med (Lausanne) 7: 248, 2020. doi:10.3389/fmed.2020.00248.

12. Adams TS, Schupp JC, Poli S, Ayaub EA, Neumark N, Ahangari F, Chu SG, Raby BA, Delulius G, Januszky M, Duan Q, Arnett HA, Siddiqui A, Washko GR, Homr R, Yan X, Rosas IO, Kaminski N. Single-cell RNA-seq reveals ectopic and aberrant lung-resident cell populations in idiopathic pulmonary fibrosis. Sci Adv 6: eaaz983, 2020. doi:10.1126/sciadv.aaz983.

13. Wolf FA, Angerer P, Theis FJ. SCANPY: large-scale single-cell gene expression data analysis. Genome Biol 19: 15, 2018. doi:10.1186/s13059-017-1382-0.

14. Ding J, Aronow BJ, Kaminski N, Kitzmiller J, Whitsett JA, Bar- Joseph Z. Reconstructing differentiation networks and their regulation from time series single-cell expression data. Genome Res 28: 383–395, 2018. doi:10.1101/gr.225979.117.

15. Sheridan JA, Zago M, Nair P, Rico de Souza A, Gallouzi IE, Rousseau S, Di Marco S, Hamid Q, Eidelman DH, Bagli BJ. Aryl hydrocarbon receptor-dependent retention of nuclear RelB and suppression of intercellular adhesion molecule-1 (ICAM-1). Toxicol Sci 140: 204–223, 2014. doi:10.1093/txsci/kfu068.

16. Zago M, Sheridan JA, Nair P, Rico de Souza A, Gallouzi IE, Rousseau S, Di Marco S, Hamid Q, Eidelman DH, Bagli BJ. Aryl hydrocarbon receptor-dependent retention of nuclear HIF suppresses cigarette smoke-induced cyclooxygenase-2 expression independent of DNA-binding. PLoS One 8: e74953, 2013. doi:10.1371/journal.pone.0074953.

17. Jacobs M, Van Eckhoutte HP, Wijnant SRA, Janssen W, Joos GF, Brusselle GG, Bracke KR. Increased expression of ACE2, the SARS-CoV-2 entry receptor, in alveolar and bronchial epithelium of smokers and COPD subjects. Eur Respir J 56: 2002378, 2020. doi:10.1183/13993003.02378-2020.

18. Bagli CJ, Sime PJ, Chong YH, Hsieh WS, Zhou Y, Zhang X, Yang F. Protective effect of angiotensin (I-7) on silicotic fibrosis in rats. Biomed Environ Sci 32: 419–426, 2019. doi:10.3967/bes.2019.057.

19. Bagli CJ, Sime PJ, Hogg J, Madani R, Quattrini B, Kudlow P, Cameron J. Alternative roles of STAT3 and MAPK signaling pathways in the MMPs activation and progression of lung injury induced by cigarette smoke exposure. AJP Lung Cell Mol Physiol 325: 262–285, 2018. doi:10.1152/ajplung.00306.2005.

20. Bagli CJ, Sime PJ. The aryl hydrocarbon receptor attenuates tobacco smoke-induced cyclooxygenase-2 and prostaglandin production in lung fibroblasts through regulation of the NF-kappaB family member RelB. J Biol Chem 283: 28944–28957, 2008. doi:10.1074/jbc.M800685200.