INFECTION OF HUMAN AIRWAY EPITHELIA BY SARS CORONAVIRUS IS ASSOCIATED WITH ACE2 EXPRESSION AND LOCALIZATION

Hong Peng Jia, Dwight C. Look, Melissa Hickey, Lei Shi, Lecia Pewe, Jason Netland, Michael Farzan, Christine Wohlford-Lenane, Stanley Perlman, and Paul B. McCray, Jr.*

1. INTRODUCTION

Severe acute respiratory syndrome (SARS) emerged as a regional and global health threat in 2002–2003 resulting in approximately 800 deaths.[1] An intense, cooperative worldwide effort rapidly led to the identification of the disease causing agent as a novel coronavirus (SARS-CoV)[2,3] and the subsequent complete sequencing of the viral genome. Although limited human pathological studies demonstrate that the respiratory tract is a major site of SARS-CoV infection and morbidity, little is known regarding the initial steps in SARS-CoV-host cell interactions in the respiratory tract, such as the cell types in which primary viral infection and replication occur.

Angiotensin converting enzyme 2 (ACE2) was identified as a receptor for both SARS-CoV[4] and NL63.[5] ACE2 is a membrane-associated aminopeptidase expressed in vascular endothelia, renal and cardiovascular tissues, and epithelia of the small intestine, and testes.[6,7] A region of the extracellular portion of ACE2 that includes the first α-helix, and lysine 353 and proximal residues of the N-terminus of β-sheet 5 interacts with high affinity to the receptor binding domain of the SARS-CoV S glycoprotein.[8] Several unanswered questions remain regarding ACE2 expression in human respiratory epithelia and its role as a receptor for SARS-CoV, including identification of the specific epithelial cell types expressing ACE2, the polarity of ACE2 expression, and whether SARS-CoV infection of respiratory epithelia is ACE2-dependent.

* Hong Peng Jia, Dwight C. Look, Melissa Hickey, Lei Shi, Lecia Epping, Jason Netland, Christine Wohlford-Lenane, Stanley Perlman, Paul B. McCray, Jr., University of Iowa, Iowa City, Iowa 52242. Michael Farzan, Harvard Medical School, Southborough, Massachusetts 01772.

2. ACE2 EXPRESSION IN AIR-LIQUID INTERFACE CULTURED AIRWAY EPITHELIA IS POLARIZED

To try to answer some of these remaining questions, we use an air-liquid interface culture model of primary human airway epithelia in our studies. Airway epithelial cells of trachea or bronchi from human donors were grown on collagen coated porous filters with the apical side in contact with air and the basolateral side immersed in culture medium. Over 2 weeks, the cells polarize, develop transepithelial resistance and some become ciliated, signs of differentiation. TEM images show that these cells form a thin layer of secretions at the apical side, creating an air-liquid interface. So this pseudostratified epithelium closely mimics the *in vivo* situation.[9]

To understand the potential role for ACE2 as the SARS-CoV receptor in the respiratory tract, we first looked for evidence of ACE2 protein expression in human lung tissue by Western blot. ACE2 was identified in lysates from human conducting airway and distal lung tissues, but this result did not indicate which cell types expressed ACE2 (data not shown). We next evaluated ACE2 protein expression in well-differentiated primary cultures of airway epithelia by immunohistochemistry. The signal for ACE2 was more abundant on the apical rather than the basolateral surface (Figure 1A). Furthermore, the signal intensity was strongest on ciliated cells, as demonstrated by co-localization with beta-tubulin IV, a marker of cilia,[11] suggesting that ciliated cells express ACE2 abundantly. To confirm a polar distribution of ACE2 in differentiated epithelia, selective apical or basolateral surface biotinylation with subsequent immunoprecipitation was performed (Figure 1B). Western blot analysis of immunoprecipitated proteins confirmed that ACE2 is expressed in greater abundance on the apical surface of conducting airway epithelia, although a weak ACE2 signal was also detected basolaterally. In contrast, ErbB2 was more abundant on the basolateral surface as previously reported, confirming selective biotinylation.[10]

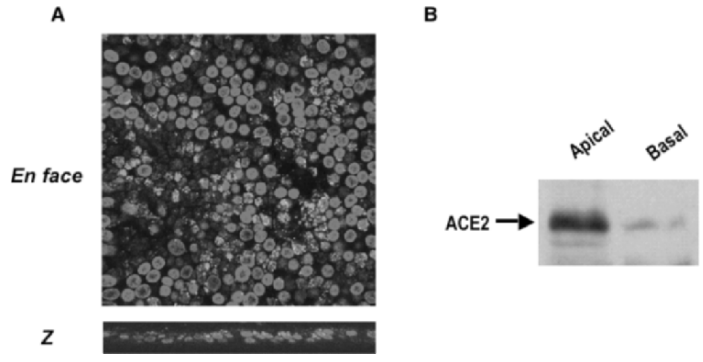

Figure 1. ACE2 is expressed in human airway epithelia. (A) ACE2 protein location in polarized human airway epithelia was determined using immunofluorescence staining for ACE2 (green) and the nucleus (ethidium bromide, red). Confocal fluorescence photomicroscopic images are presented *en face* (top) and from vertical sections in the z axis (bottom). (B) ACE2 protein location in polarized human airway epithelia was determined by selective apical or basolateral biotinylation, immunoprecipitation of biotinylated surface proteins, and immunoblat analysis for ACE2. (See color plate).

3. ACE2 EXPRESSION DEPENDS ON STATE OF CELL DIFFERENTIATION

Because results from polarized epithelia suggested that ACE2 expression might depend on the state of cellular differentiation, we compared the apical surface morphology of well-differentiated epithelia with that of well-differentiated cells grown with media present on their apical surface for 7 days to promote de-differentiation. Importantly, submersion of the apical surface of polarized cells caused loss of cilia and markedly diminished expression of ACE2 mRNA and protein (Figure 2A–C). In contrast with results in polarized epithelia, poorly differentiated primary human tracheobronchial epithelia or A549 cells grown on tissue culture plastic expressed little ACE2 mRNA or protein. Notably, foxj1, a transcription factor expressed in well-differentiated ciliated epithelia was also coordinately expressed with ACE2, indicating that ACE2 positively correlates with the state of epithelial differentiation. This raised the question of whether foxj1 might regulate ACE2 expression in airway epithelia. Primary tracheobronchial cells grown in submersion culture were transduced with an adenoviral vector expressing ACE2, a negative control β-galactosidase, or foxj1. Only transduction with the ACE2 vector conferred ACE2 expression, suggesting that foxj1 alone is not sufficient enough to regulate ACE2 expression in airway epithelia.

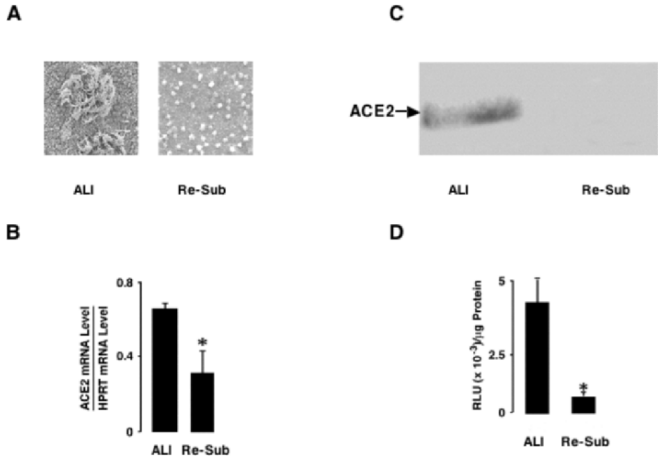

Figure 2. ACE2 expression is associated with airway epithelial cell differentiation. (A) Ciliated epithelial cell differentiation in cultures of primary airway epithelial cells under air-liquid interface or resubmerged conditions was verified by SEM of the apical epithelial surface. (B) ACE2 mRNA levels determined using realtime RT-PCR analysis of samples from differentiating air-liquid interface (ALI), or resubmerged (Re-sub) conditions. Values are expressed as mean mRNA level compared with control hypoxanthine phosphoribosyltransferase (HPRT) mRNA level ± S.D.; asterisk indicates a significant difference in mRNA levels between air-liquid interface and resubmerged conditions. (C) ACE2 protein levels determined using immunoblot of extracts from differentiating air-liquid interface (ALI), or resubmerged (Re-sub) conditions. (D) β-galactosidase levels determined in primary human airway epithelia cultured under ALI or resubmerged conditions that were infected from the apical with SARS-S protein pseudotyped FIV.

4. POLARIZATION OF S PROTEIN PSEUDOTYPED FIV VIRAL ENTRY

To evaluate the polarity of entry of the SARS-CoV in airway epithelia, we prepared feline immunodeficiency virus (FIV) virions pseudotyped with SARS S protein, and this vector was used to contrast the efficiency of entry in A549 cells, poorly differentiated (submerged) human airway epithelia, and well-differentiated epithelia. Only well-differentiated epithelial cells showed significant β-galactosidase expression following transduction (Figure 2D). The ACE2 dependence of transduction with SARS S protein FIV pseudotyped virions, which also express β-galactosidase, was first evaluated on 293 cells with or without co-transfection with human ACE2 cDNA. The result indicated that 293 cell transduction with this vector was almost completely ACE2-dependent. To further evaluate the ACE2 dependence of human airway epithelia for SARS-CoV, we transduced poorly differentiated A549 cells and submerged primary airway epithelia that do not express constitutive ACE2 with increasing MOIs of an adenoviral vector expressing human ACE2. After 48 hr, SARS-CoV S protein pseudotyped FIV was applied to the apical surface. The results showed that there was an inoculum-dependent increase in transduction of the ACE2 complemented cells. We next applied the pseudotyped virus to the apical or basolateral surfaces of well-differentiated primary cultures of human airway epithelia to investigate if the virus preferentially entered from one cell surface. Two days later the cells were harvested and entry evaluated by β-galactosidase activity. Results indicated that the S protein pseudotyped virions transduced human airway epithelia more efficiently when applied from the apical rather than the basolateral surface. This pattern of entry correlates with ACE2 expression on polarized cells. As a control, FIV pseudotyped with the VSV-G envelope entered polarized cells better from the basolateral surface.

5. SARS-CoV INFECTION OF AIRWAY EPITHELIA

We also conducted selected experiments using wild-type SARS-CoV (Urbani strain) and evaluated the ability of SARS-CoV to infect multiple human airway epithelial cell culture models. Under BSL3 containment we applied the virus to A549 cells, poorly differentiated (submerged) primary cultures of airway epithelia, or well-differentiated (air-liquid interface) human airway epithelia. A549 and hTBE cells cultured under submerged conditions expressed little detectable SARS-CoV N or S gene mRNA. In contrast, in well-differentiated cells infected with SARS-CoV from the apical surface, the N and S gene mRNAs were detected at high levels. We confirmed that the gene products detected in the real time RT-PCR assays were generated from new SARS-CoV mRNA templates rather than the viral genome by verifying the appropriate size of the amplified products. These results indicated that SARS-CoV infects undifferentiated human airway epithelial cells poorly or not at all, while well-differentiated conduction airway epithelia are susceptible.

By applying the virus to the apical surface of well-differentiated human airway epithelia for 30 min and then measuring the release of virus 24 hr later by tittering the virus, we documented that SARS-CoV productively infects human airway epithelia. The results indicate that following apical application of SARS-CoV a productive infection occurred and virus was preferentially released apically. We confirmed SARS-CoV

infection of polarized epithelia by immunostaining cells for the SARS-CoV nsp1 protein 24 hr following infection.

6. CONCLUSIONS

Our studies revealed the novel observation that SARS-CoV infection of human airway epithelia is dependent upon the state of epithelial differentiation and ACE2 mRNA and protein expression. ACE2 is more abundantly expressed on the apical surface of polarized epithelia. The predominant apical distribution of ACE2 suggests that the enzyme may be available to cleave peptides at the mucosal surface of the airway but the native substrates in the lung have not yet been identified. We show for the first time that well-differentiated cells support viral replication with viral entry and egress occurring from the apical surface. Thus, SARS-CoV preferentially infects well-differentiated epithelial cells expressing ACE2. Because ACE2 is also the receptor for the coronavirus NL63,[4] these findings are relevant to the biology of infection with this more common human pathogen.

Human ACE2 appears necessary and sufficient to serve as a receptor for SARS-CoV.[5] Our findings suggest that the epithelium of the conducting airways, the major site of respiratory droplet deposition, supports the replication of SARS-CoV. The observation that ACE2 complementation of poorly differentiated epithelia enhanced transduction with S protein pseudotyped virions in a dose-dependent manner further supports its role as a receptor. Although both DC-SIGN (CD209) and DC-SIGN (L-SIGN, CD209L) can enhance SARS-CoV infection of ACE2 expressing cells, these proteins are not sufficient to support infection in the absence of ACE2.[12, 13] Several recent reports using SARS-CoV or retroviral vectors pseudotyped with SARS S protein[14] indicated that human airway epithelial cell lines were poorly transduced, an unexpected finding that raised questions regarding the ability of respiratory epithelia to support SARS-CoV infection. The present studies help explain these findings. Because SARS-CoV infection of airway epithelia is ACE2-dependent and ACE2 expression is greatest in well-differentiated cells, the low transduction efficiencies of non-polarized, poorly differentiated cells are not unanticipated.

In the setting of a productive infection of conducting airway epithelia, the apically released SARS-CoV might be removed by mucociliary clearance and gain access to the gastrointestinal tract. SARS-CoV infects cells in the gastrointestinal tract and diarrhea is a clinical sign commonly observed in patients with SARS.[1] Furthermore, the preferential apical exit pathway of virions would favor spread of infection along the respiratory tract. While not a focus of our study, pathologic data indicate that SARS-CoV infects type II pneumocytes. Infection and release of virus in this compartment with its close proximity to the pulmonary capillary bed might allow systemic spread of virus to distant organs, especially in the context of inflammation and alveolar capillary leak.

In conclusion, studies in models of human airway epithelial differentiation and polarity reveal that SARS-CoV infects well-differentiated cells from the apical surface and preferentially exits from the apical side. These findings should also apply to the entry of NL63 in human airway epithelia. ACE2 expression in airway epithelia appears to be both necessary and sufficient for SARS-CoV infection. Airway epithelial expression of ACE2 is dynamic and associated with cellular differentiation, a finding that may underlie susceptibility to infection. The apical expression of ACE2 on epithelia indicates that this

coronavirus receptor is accessible for topical application of receptor antagonists or inhibitors. To date, the factors regulating ACE2 expression have not been identified. Future studies of the ACE2 promoter and gene expression associated with cell differentiation may reveal regulators of ACE2 expression and subsequent SARS-CoV and NL63 susceptibility.

7. REFERENCES

1. C. A. Donnelly, A. C. Ghani, G. M. Leung, A. J. Hedley, C. Fraser, S. Riley, L. J. Abu-Raddad, L. M. Ho, T. Q. Thach, P. Chau, K. P. Chan, T. H. Lam, L. Y. Tse, T. Tsang, S. H. Liu, J. H. Kong, E. M. Lau, N. M. Ferguson, and R. M. Anderson, Epidemiological determinants of spread of causal agent of severe acute respiratory syndrome in Hong Kong, *Lancet* **361**, 1761-1766 (2003).
2. S. M. Poutanen, D. E. Low, B. Henry, S. Finkelstein, D. Rose, K. Green, R. Tellier, R. Draker, D. Adachi, M. Ayers, A. K. Chan, D. M. Skowronski, I. Salit, A. E. Simor, A. S. Slutsky, P. W. Doyle, M. Krajden, M. Petric, R. C. Brunham, and A. J. McGeer, Identification of severe acute respiratory syndrome in Canada, *N. Engl. J. Med.* **348**, 1995-2005 (2003).
3. T. J. Franks, P. Y. Chong, P. Chui, J. R. Galvin, R. M. Lourens, A. H. Reid, E. Selbs, C. P. McEvoy, C. D. Hayden, J. Fukuoka, J. K. Taubenberger, and W. D. Travis, Lung pathology of severe acute respiratory syndrome (SARS): a study of 8 autopsy cases from Singapore, *Hum. Pathol.* **34**, 743-748 (2003).
4. W. Li, M. J. Moore, N. Vasilieva, J. Sui, S. K. Wong, M. A. Berne, M. Somasundaran, J. L. Sullivan, K. Luzuriaga, T. C. Greenough, H. Choe, and M. Farzan, Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus, *Nature* **426**, 450-454 (2003).
5. H. Hofmann, P. Krzysztof, L. van der Hoek, M. Geier, B. Berkhout, and S. Pohlmann, Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry, *Proc. Natl. Acad. Sci. USA* (2005).
6. M. Donoghue, F. Hsieh, E. Baronas, K. Godbout, M. Gosselin, N. Stagliano, M. Donovan, B. Woolf, K. Robison, R. Jeyaseelan, R. E. Breitbart, and S. Acton, A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9, *Circ. Res.* **87**, E1-9 (2000).
7. I. Hamming, W. Timens, M. L. Bulthuis, A. T. Lely, G. J. Navis, and H. van Goor, Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis, *J. Pathol.* **203**, 631-637 (2004).
8. W. Li, C. Zhang, J. Sui, J. H. Kuhn, M. J. Moore, S. Luo, S. K. Wong, I. C. Huang, K. Xu, N. Vasilieva, A. Murakami, Y. He, W. A. Marasco, Y. Guan, H. Choe, and M. Farzan, Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2, *EMBO J.* (2005).
9. P. H. Karp, T. O. Moninger, S. P. Weber, T. S. Nesselhauf, J. L. Launspach, J. Zabner, and M. J. Welsh, An in vitro model of differentiated human airway epithelia. Methods for establishing primary cultures, *Methods Mol. Biol.* **188**, 115-137 (2002).
10. P. D. Vermeer, L. A. Einwalter, T. O. Moninger, T. Rokhlina, J. A. Kern, J. Zabner, and M. J. Welsh, Segregation of receptor and ligand regulates activation of epithelial growth factor receptor, *Nature* **422**, 322-326 (2003).
11. D. C. Look, M. J. Walter, M. R. Williamson, L. Pang, Y. You, J. N. Sreshta, J. E. Johnson, D. S. Zander, and S. L. Brody, Effects of paramyxoviral infection on airway epithelial cell Foxj1 expression, ciliogenesis, and mucociliary function, *Am. J. Pathol.* **159**, 2055-2069 (2001).
12. S. A. Jeffers, S. M. Tusell, L. Gillim-Ross, E. M. Hemmila, J. E. Achenbach, G. J. Babcock, W. D. Thomas, Jr., L. B. Thackray, M. D. Young, R. J. Mason, D. M. Ambrosino, D. E. Wentworth, J. C. Demartini, and K. V. Holmes, CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus, *Proc. Natl. Acad. Sci. USA* **101**, 15748-15753 (2004).
13. A. Marzi, T. Gramberg, G. Simmons, P. Moller, A. J. Rennekamp, M. Krumbiegel, M. Geier, J. Eisemann, N. Turza, B. Saunier, A. Steinkasserer, S. Becker, P. Bates, H. Hofmann, and S. Pohlmann, DC-SIGN and DC-SIGNR interact with the glycoprotein of Marburg virus and the S protein of severe acute respiratory syndrome coronavirus, *J. Virol.* **78**, 12090-12095 (2004).
14. Y. Nie, P. Wang, X. Shi, G. Wang, J. Chen, A. Zheng, W. Wang, Z. Wang, X. Qu, M. Luo, L. Tan, X. Song, X. Yin, M. Ding, and H. Deng, Highly infectious SARS-CoV pseudotyped virus reveals the cell tropism and its correlation with receptor expression, *Biochem. Biophys. Res. Commun.* **321**, 994-1000 (2004).