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Short Communication

Analysis of ACE2 in polarized epithelial cells: surface expression and function as receptor for severe acute respiratory syndrome-associated coronavirus

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The primary target of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) is epithelial cells in the respiratory and intestinal tract. The cellular receptor for SARS-CoV, angiotensin-converting enzyme 2 (ACE2), has been shown to be localized on the apical plasma membrane of polarized respiratory epithelial cells and to mediate infection from the apical side of these cells. Here, these results were confirmed and extended by including a colon carcinoma cell line (Caco-2), a lung carcinoma cell line (Calu-3) and Vero E6 cells in our analysis. All three cell types expressed human ACE2 on the apical membrane domain and were infected via this route, as determined with vesicular stomatitis virus pseudotypes containing the S protein of SARS-CoV. In a histological analysis of the respiratory tract, ACE2 was detected in the trachea, main bronchus and alveoli, and occasionally also in the small bronchi. These data will help us to understand the pathogenesis of SARS-CoV infection.

Epithelia are a primary barrier to infection by microorganisms entering their host via body cavities such as the respiratory or intestinal tract (reviewed by Compans & Herrler, 2005). Epithelial cells are organized in a polarized fashion that involves the separation of the plasma membrane into an apical and a basolateral domain. The polarity of these cells affects both the early and late stages of infection, i.e. viruses may enter into and exit from a cell either via the apical membrane facing the external environment or via the basolateral membrane directed to the internal milieu of the organism. An important determinant of the virus infection is the presence of suitable receptors on the cell surface that allow attachment to and penetration through the plasma membrane. For viruses entering their host via the respiratory or gastrointestinal route, infection is understood most easily when the virus receptor is expressed on the apical surface.

The primary target of the coronavirus associated with severe acute respiratory syndrome (SARS-CoV) is the respiratory tract. In addition to respiratory complications, some patients show intestinal symptoms, indicating that not only the respiratory but also the intestinal epithelium is susceptible to infection. It has been shown recently that the receptor for SARS-CoV, angiotensin-converting enzyme 2 (ACE2; Li *et al.*, 2003; Wang *et al.*, 2004), is localized on and mediates infection through the apical plasma membrane of respiratory epithelial cells (Jia *et al.*, 2005; Sims *et al.*, 2005; Tseng *et al.*, 2005). On the other hand, ACE2 has been reported to be absent from enterocytes of the colon (Hamming *et al.*, 2004), despite active replication of SARS-CoV in this portion of the intestine (Leung *et al.*, 2003).

To determine whether epithelial cells of different origin differ in the expression of ACE2, we included in our analysis three cell lines that form a highly polarized epithelial monolayer when grown on microporous filters: (i) Calu-3 (human lung carcinoma cells), (ii) Caco-2 (human colon carcinoma cells) and (iii) Vero E6 (monkey kidney cells) commonly used to propagate SARS-CoV. At 5 days post-seeding, the polarity of the monolayer was verified by measurement of transepithelial resistance using a voltohmmeter (Millipore) and cells were subjected to indirect immunofluorescence analysis. Samples were fixed with 3 % (w/v) paraformaldehyde

in PBS for 20 min, quenched in 0·1 M glycine for 5 min and permeabilized with 0·2 % Triton X-100 for 5 min in PBS. Incubation with the primary antibody (anti-ACE2, R&D Systems; anti-gp58, kindly provided by Doris Meder, Max-Planck-Institut für molekulare Zellbiologie und Genetik, Dresden, Germany; both antibodies diluted in 1 % BSA) for 2 h in a humidified chamber was followed by a 2 h incubation in the dark with the appropriate fluorescein isothiocyanate-conjugated secondary antibody (Sigma). Cells were mounted in Mowiol and examined with a fluorescence microscope equipped for laser-scanning confocal light microscopy (DM IRB/E; Leica). Optical sections were recorded by using a 63 × oil-immersion objective. As shown in Fig. 1, confocal laser-scanning microscopy demonstrated that ACE2 was detected on the surface of some Calu-3 cells by horizontal laser scanning. Vertical laser scanning confirmed previous reports that ACE2 was located exclusively in the apical plasma membrane of Calu-3 cells (Tseng *et al.*, 2005). Glycoprotein gp58, a basolateral marker protein, was expressed by Calu-3 cells, forming a ring along the borders of the cells. It was distributed predominantly in the lateral plasma membrane (Fig. 1). A significantly larger number of Caco-2 cells showed surface expression of ACE2 compared with Calu-3 cells and the protein was localized exclusively on the apical plasma membrane (Fig. 1). The strongest expression of endogenous ACE2 was detected on Vero E6 cells. Although these cells only developed a low electrical resistance, ACE2 showed a polarized localization; in agreement with the two other epithelial cell lines, ACE2 was restricted to the apical surface of Vero E6 cells. These results were confirmed by domain-selective surface biotinylation. ACE2 was labelled only when the membrane-impermeable biotinylating reagent was added to the apical side of the monolayer. In agreement with the fluorescence analysis, clear ACE2 bands were detected in the case of Caco-2 and Vero E6 cells, whereas only a faint band was visible when Calu-3 cells were analysed (data not shown).

To analyse the infection of polarized cells by SARS-CoV, we used vesicular stomatitis virus (VSV) pseudotypes. These defective virions lack the gene for the surface glycoprotein G of VSV (VSV-ΔG). They can form infectious virions only if the G protein or a foreign viral surface glycoprotein is provided *in trans*, e.g. by transfection of cells. VSV pseudotypes were generated as described previously (Hanika *et al.*, 2005). We used this approach to obtain pseudotypes containing the S protein of SARS-CoV (VSV-ΔG-S). Similar to lentiviral pseudotypes, highest infectivity was observed when the cytoplasmic tail of the S protein was truncated by

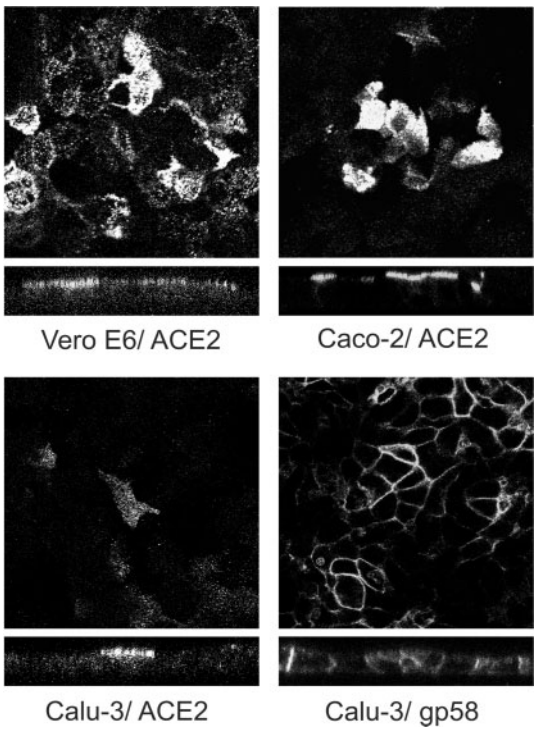

Fig. 1. Localization of ACE2 on polarized epithelial cells by confocal immunofluorescence microscopy. Vero E6, Caco-2 or Calu-3 cells were seeded on polycarbonate filters. At 5 days post-seeding, cells were stained for surface expression of ACE2. Control cells were stained for gp58, which served as a basolateral marker. Both horizontal (top) and vertical (bottom) laser scanning are shown.

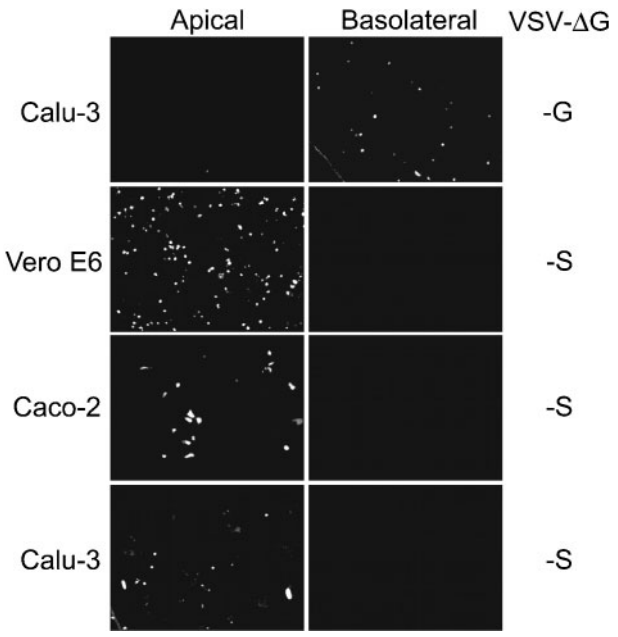

Fig. 2. Infection of polarized epithelial cells by VSV pseudotypes mediated by the S protein of SARS-CoV. Vero E6, Caco-2 or Calu-3 cells were seeded on polycarbonate filters. At 5 days post-seeding, cells were infected from either the apical or the basolateral side with VSV pseudotypes carrying either the S protein of SARS-CoV (VSV-ΔG-S) or the G protein of VSV (VSV-ΔG-G). Fluorescence of GFP expressed from the pseudotype genome was used to visualize infected cells.

deletion of the 18 C-terminal amino acids (Nie *et al.*, 2004). The requirement for the C-terminal truncation is not related to intracellular retention, which has been observed with other coronavirus S proteins, but not with SARS-CoV (Schwegmann-Wessels *et al.*, 2004). As the VSV-G gene had been replaced by the green fluorescent protein (GFP) gene in the pseudotype genomes, GFP expression could be used to detect infected cells by fluorescence microscopy. Highly polarized Vero E6, Caco-2 and Calu-3 cells grown on 12 mm diameter filters were infected with VSV pseudotypes (1×10^5 infectious units) from either the apical or the basolateral side. Infectious units were determined by counting GFP-expressing cells; the pseudotypes applied to the filter-grown cells corresponded to an m.o.i. of 0·25–0·5. At 16 h post-infection, cells were fixed with paraformaldehyde (3 %, 20 min). As shown in Fig. 2, pseudotypes containing the VSV-G protein infected Calu-3 cells preferentially from the basolateral plasma membrane. This result served as a control that the membrane filter does not prevent basolateral infection. When VSV pseudotypes containing the S protein of SARS-CoV were used for infection, a different picture was observed. With all three cell lines (Vero E6, Calu-3 and Caco-2 cells), infection was initiated successfully only from the apical and not from the basolateral side (Fig. 2). The highest number of infected cells was detected with Vero E6 cells followed by Caco-2 cells, whilst the lowest infection rate was obtained with Calu-3 cells. This difference in efficiency of infection corresponded with the different expression levels of ACE2 in these cells. Our results extend previous reports by applying the analysis to epithelial cells from different origins, including the human colon. Our control infections showed that the membrane pores did not prevent basolateral infection.

In order to understand the pathogenesis of SARS-CoV infection, it is important to examine the expression of ACE2 in the respiratory tract. Therefore, human respiratory tissues ($n=5$) were collected and analysed for expression of ACE2. The patients included were free of tumour ($n=1$) or had died from causes unrelated to pulmonary diseases (coronary failure, $n=2$; accidents, $n=2$). Cryosections of 6 μm on poly-L-lysine-coated slides were pre-incubated with 10 % human serum to reduce non-specific staining. After incubation with an antibody directed against ACE2, subsequent staining was performed by using secondary antibody (rabbit anti-goat; Dako) and detection antibody (anti-rabbit alkaline phosphatase anti-alkaline phosphatase; Sigma) with FastBlue as substrate. The slides were counterstained with haematoxylin (Merck). A weak apical expression of ACE2 was found in some of the epithelial cells of the trachea

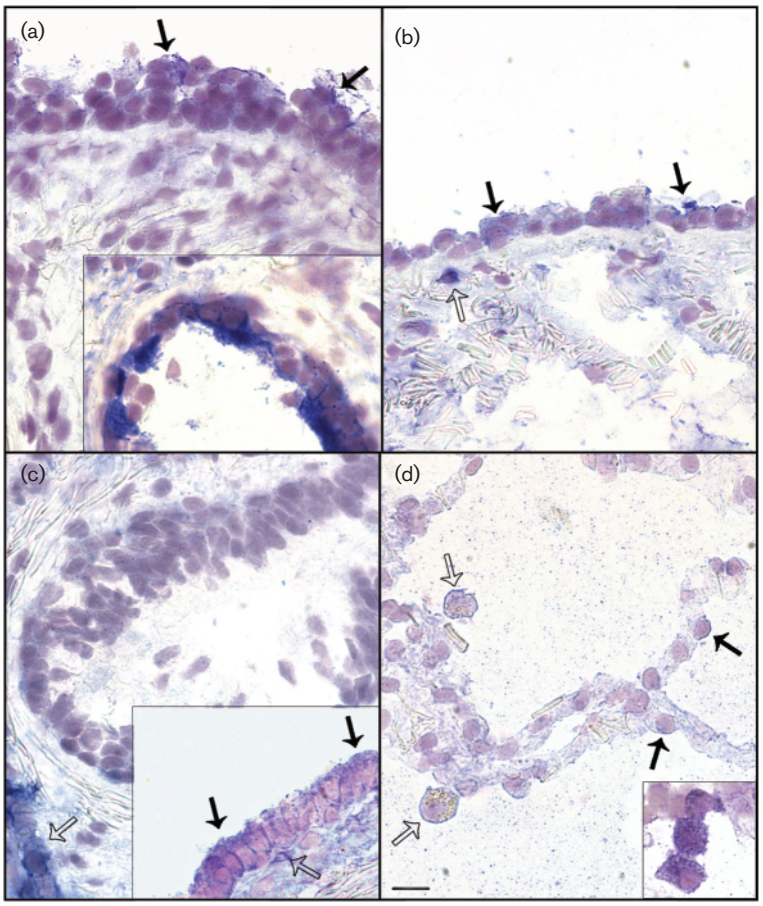

Fig. 3. ACE2 expression in the respiratory tract. All tissues were from a patient who had died from coronary failure, except for the sample shown in the insert of (c), which was obtained from surgical-resection material. Filled arrows point to positively stained epithelial cells and open arrows to positive non-epithelial cells, mostly leukocytes. All photographs, including inserts, were recorded at 400× magnification. (a) Tracheal epithelium and epithelium of the tracheal glands (insert). (b) Epithelium of the main bronchus. (c) Epithelium of the lower bronchi. (d) Alveolar epithelium with the insert showing the staining of the surface of some alveolar macrophages (thick cryosection, 10 μm). Bar, 40 μm.

(Fig. 3a), of the main bronchus (Fig. 3b) and of the alveoli (Fig. 3d). Strong expression of ACE2 was observed on the epithelium of almost all tracheal glands (Fig. 3a, insert). No ACE2 was detected in the lower bronchi of any of the tissues collected post-mortem. However, an almost-continuous staining of some of the bronchi was found when resection material was analysed (Fig. 3c, insert). Besides epithelial cells, some non-epithelial cells in the mucosa of the trachea and main bronchus also showed positive staining for ACE2. These results indicated that ACE2 is present on epithelial cells in several parts of the respiratory tract. Therefore, the tracheal or bronchial epithelium may be susceptible to infection by SARS-CoV. Such primary sites of infection possibly facilitate the spread of virus to the alveoli, where the main damage occurs. Staining of the lower bronchi for ACE2 was successful in only a few samples. This result suggested that there may be variations in the expression levels of ACE2. The expression of this metalloprotease is possibly induced by environmental stimuli, such as microbial infections or inflammatory processes in the lung. This explanation may also apply to intestinal infections, which are known to occur in the colon (Leung *et al.*, 2003), although ACE2 has been reported to be absent from the respective enterocytes (Hamming *et al.*, 2004). It is possible that expression in the colon is induced by cellular or environmental stimuli. On the other hand, ACE2 is abundantly present on the enterocytes of the small intestine (Hamming *et al.*, 2004). Nevertheless, intestinal symptoms occur only in some patients. This may reflect the difficulties encountered by the virus in accessing the intestinal epithelium, which is covered by a thick layer of mucus. This has been suggested by results obtained with *Transmissible gastroenteritis virus* (TGEV), an enteropathogenic porcine coronavirus, which requires two binding activities to be enteropathogenic. TGEV uses aminopeptidase N, another metalloprotease, as a receptor (Delmas *et al.*, 1992). Although this protein is present in intestinal brush-border membranes, efficient intestinal infection is observed only with strains that have a sialic acid-binding activity (Schultze *et al.*, 1996; Krempl *et al.*, 1997). Binding to sialic acid may allow the interaction with sialic acid-rich mucins and thus facilitate penetration of the mucus layer (Schwegmann-Wessels *et al.*, 2002, 2003). SARS-CoV lacks a sialic acid-binding activity. This may explain why this virus is mainly a respiratory and not an intestinal pathogen. Finally, it should be noted that another human coronavirus, NL63, also uses ACE2 as a cellular receptor (Hofmann *et al.*, 2005). Therefore, our findings are also relevant for infections by this virus.

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