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ScienceDirect
Virus Research 133 (2008) 33–44
Virus Research
www.elsevier.com/locate/virusres

SARS-CoV replication and pathogenesis in an *in vitro* model of the human conducting airway epithelium

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Available online 23 April 2007

Abstract

SARS coronavirus (SARS-CoV) emerged in 2002 as an important cause of severe lower respiratory tract infection in humans and *in vitro* models of the lung are needed to elucidate cellular targets and the consequences of viral infection. The severe and sudden onset of symptoms, resulting in an atypical pneumonia with dry cough and persistent high fever in cases of severe acute respiratory virus brought to light the importance of coronaviruses as potentially lethal human pathogens and the identification of several zoonotic reservoirs has made the reemergence of new strains and future epidemics all the more possible. In this chapter, we describe the pathology of SARS-CoV infection in humans and explore the use of two models of the human conducting airway to develop a better understanding of the replication and pathogenesis of SARS-CoV in relevant *in vitro* systems. The first culture model is a human bronchial epithelial cell line Calu-3 that can be inoculated by viruses either as a non-polarized monolayer of cells or polarized cells with tight junctions and microvilli. The second model system, derived from primary cells isolated from human airway epithelium and grown on Transwells, form a pseudostratified mucociliary epithelium that recapitulates the morphological and physiological features of the human conducting airway *in vivo*. Experimental results using these lung epithelial cell models demonstrate that in contrast to the pathology reported in late stage cases SARS-CoV replicates to high titers in epithelial cells of the conducting airway. The SARS-CoV receptor, human angiotensin 1 converting enzyme 2 (hACE2), was detected exclusively on the apical surface of cells in polarized Calu-3 cells and human airway epithelial cultures (HAE), indicating that hACE2 was accessible by SARS-CoV after lumenal airway delivery. Furthermore, in HAE, hACE2 was exclusively localized to ciliated airway epithelial cells. In support of the hACE2 localization data, the most productive route of inoculation and progeny virion egress in both polarized Calu-3 and ciliated cells of HAE was the apical surface suggesting mechanisms to release large quantities of virus into the lumen of the human lung. Preincubation of the apical surface of cultures with antisera directed against hACE2 reduced viral titers by two logs while antisera against DC-SIGN/DC-SIGNR did not reduce viral replication levels suggesting that hACE2 is the primary receptor for entry of SARS-CoV into the ciliated cells of HAE cultures. To assess infectivity in ciliated airway cultures derived from susceptible animal species we generated a recombinant SARS-CoV by deletion of open reading frame 7a/7b (ORF 7a/7b) and insertion of the green fluorescent protein (GFP) resulting in SARS-CoV GFP. SARS-CoV GFP replicated to similar titers as wild type viruses in Vero E6, MA104, and CaCo2 cells. In addition, SARS-CoV replication in airway epithelial cultures generated from Golden Syrian hamster tracheas reached similar titers to the human cultures by 72 h post-infection. Efficient SARS-CoV infection of ciliated cell-types in HAE provides a useful *in vitro* model of human lung origin to study characteristics of SARS-CoV replication and pathogenesis.

Keywords: Human airway epithelia; SARS-CoV; Coronavirus replication; SARS-CoV GFP; Coronavirus pathogenesis

1. Introduction

The importance of human coronaviruses (HCoV) as pathogens that produce severe human respiratory diseases has been greatly emphasized with the identification of the SARS-CoV and relevant model systems are needed to elucidate the underlying molecular mechanisms governing coronavirus pathogenesis and virulence in the human lung. SARS-CoV infection is an attractive model for HCoV infection as it produces severe disease in the human lung, replicates efficiently *in vitro*, reverse genetics systems are available to identify the genetic determinants governing pathogenesis and virulence, and a variety of animal models are under development (Almazan et al., 2006; Lawler et al., 2006; Martina et al., 2003; McCray et al., 2006; Osterhaus et al., 2004; Roberts et al., 2005a,b; Tseng et al., 2006; Yount et al., 2003). In this article we will provide a brief review of the pathology of SARS-CoV infection as well as

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doi:10.1016/j.virusres.2007.03.013

reviewing research examining human coronavirus infection of *in vitro* models of the human conducting airway. Finally, we will extend current data on the characterization of SARS-CoV infection of an *in vitro* culture system of human airway epithelium (HAE) that recapitulates the morphological and physiological features of the human conducting airway *in vivo* to determine whether infection and spread of SARS-CoV throughout the ciliated conducting airway may be a valid model for understanding the pathogenesis of SARS-CoV lung disease.

SARS-CoV caused about 8000 cases and ~800 deaths worldwide with a ~10% overall mortality rate prior to successful containment of the epidemic. Mortality rates following SARS-CoV infection approached <1% under 24 years of age, 6% for ages 15–44, 15% for ages 45–64 and >50% over 65, and survivors developed lung and cardiac complications (Han et al., 2003). The disease caused by this pathogenic human coronavirus is in stark contrast to that seen with other strains (229E and OC43), which produce only mild common cold symptoms, although more serious disease has been reported in infants and individuals with underlying co-morbidities (Pene et al., 2003). SARS-CoV has been isolated from humans, civet cats, raccoon dogs, swine and bats, suggesting that several animal species may function as natural reservoirs for future outbreaks (Guan et al., 2003). The Chinese horseshoe bat, which is abundant across Southeast Asia, is probably the natural reservoir for SARS-CoV (Guan et al., 2003; Lau et al., 2005; Li et al., 2005; Poon et al., 2005). Importantly, bats and bat products are used in food and traditional medicine markets in southern China and Asia and bat feces are used in traditional Chinese medicines providing a constant source of human exposure to bats and bat tissues making future SARS or SARS-like outbreaks more likely (Fujita, 1988). The SARS-CoV epidemic is the best characterized in terms of the changes that likely evolved following initial introduction of mildly virulent zoonotic strains into human populations, to moderate and highly virulent isolates that circulated around the world in less than 6 months (Chinese, 2004).

2. Clinical evidence for SARS-CoV pathogenesis

The predominant pathological features of SARS-CoV infection of the human lung include diffuse alveolar damage (DAD), atypical pneumonia with dry cough, persistent fever, progressive dyspnea and sometimes, abrupt deterioration of lung function. Major pathologic lesions include inflammatory exudation in the alveoli and interstitial tissue with hyperplasia of fibrous tissue and fibrosis. These represent major histologic changes associated with acute respiratory distress syndrome (ARDS), which has a high mortality rate and few treatment options (Cheung et al., 2004; Ksiazek et al., 2003; Kuiken et al., 2003; Nicholls and Peiris, 2005). Increasing age, male sex, presence of co-morbidity, high early viral RNA burdens, and high lactate dehydrogenase (LDH) levels are associated with greater risk of death (Chu et al., 2004; Leung and Chiu, 2004). Using fluorescence *in situ* hybridization and tissue from fatal cases of disease, virus was localized within alveolar pneumocytes (primarily type II) and within alveolar spaces. Previously, the reported receptor for SARS-CoV, the angiotensin 1 converting enzyme 2 (hACE2) had only been localized to alveolar cells in the lung although recent evidence indicates that hACE2 is present throughout the human airway epithelium suggesting additional cellular targets of infections (Jia et al., 2005). SARS-CoV specific RNA has also been localized in pulmonary macrophages although whether viable virus was present was not determined (Lu et al., 2005). In another study based on six patients who died from SARS the most pronounced morphological features were giant cell formation (predominately macrophages) and pneumocyte hyperplasia suggesting that proinflammatory cytokines released by stimulated macrophages in the alveolus were a predominant cause of pathogenesis (Nicholls et al., 2003a,b). Histological samples for determining SARS-CoV infection of airways have been less rigorously studied as pathological analyses is usually performed on late stage fatal cases (Chow et al., 2004; To et al., 2004). However, early disease noted marked bronchiolar disease with respiratory epithelial cell necrosis, loss of cilia, squamous metaplasia and intrabronchiolar fibrin deposits. In fact it has been suggested that early DAD as a result of SARS-CoV infection may initiate within the respiratory bronchioles (Franks et al., 2003; Nicholls et al., 2003a,b). It has also been hypothesized that SARS-CoV replication in the human conducting airway may be cell type restricted by expression of the receptor/co-receptor molecules hACE2 and dendritic cell-specific ICAM3-grabbing nonintegrin (DCSIGN/DCSIGNR) (Jeffers et al., 2004). DCSIGNR expression is readily detectable in alveolar type II cells the major cell type infected in the fatal cases of SARS infection. *In vitro* replication and pathogenesis models of the human conducting airway would provide a mechanism to determine all SARS coronavirus permissive cell types in the lung at earlier times post-infection and will provide clues about disease progression and potentially predict severity of disease outcome.

3. Coronavirus replication in models of the human conducting airway

3.1. SARS-CoV infection of monolayers and polarized Calu-3 cells

Several studies have been performed to identify SARS-CoV replication-competent *in vitro* models of the human conducting airway epithelium. Tseng et al. (2005) published the first SARS-CoV *in vitro* replication model using monolayers and polarized Calu-3 cells. Calu-3 cells were originally isolated from a human pulmonary adenocarcinoma and are characterized as nonciliated human lung/bronchial epithelial cells. Following inoculation of monolayers of Calu-3 cells; SARS-CoV replication was detected as early as 24 h post-infection with peak titers occurring at 48 h post-infection ($\sim 5 \times 10^6$ TCID50, Table 1). However, virus induced cytopathic effect was not detected until 8 days post-inoculation. Viral titer results were confirmed by real time PCR; with peak viral RNA detection occurring 48 h post-inoculation. Immunofluorescence assays using convalescent human serum isolated from a SARS-CoV infected patient detected SARS-CoV antigens in foci on the Calu-3 monolayers demonstrating that not all cells were productively infected. Dual labeling immunfluo-