Review

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Respiratory syncytial virus interaction with human airway epithelium

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Although respiratory syncytial virus (RSV) is a major human respiratory pathogen, our knowledge of how it causes disease in humans is limited. Airway epithelial cells are the primary targets of RSV infection *in vivo*, so the generation and exploitation of RSV infection models based on morphologically and physiologically authentic well-differentiated primary human airway epithelial cells cultured at an air–liquid interface (WD-PAECs) provide timely developments that will help to bridge this gap. Here we review the interaction of RSV with WD-PAEC cultures, the authenticity of the RSV–WD-PAEC models relative to RSV infection of human airway epithelium *in vivo*, and future directions for their exploitation in our quest to understand RSV pathogenesis in humans.

Respiratory syncytial virus and human disease

Respiratory syncytial virus (RSV) is a member of the Paramyxoviridae family and *Pneumovirus* genus [1]. It is composed of two subgroups, A and B, which often co-circulate in annual epidemics. RSV epidemics cause massive morbidity and considerable mortality worldwide [2]. It is the primary cause of hospitalization of infants in the first year of life [3]. It is a descending infection, commencing in the upper respiratory tract and descending to the lungs, that primarily infects human airway epithelium and is responsible for a spectrum of diseases ranging from rhinorrhea to life-threatening bronchiolitis and pneumonia [2]. Infants with underlying conditions, such as prematurity (<35 weeks' gestation), bronchopulmonary dysplasia, and congenital heart disease, are particularly at risk of severe disease and death from RSV infection [4,5]. Recurrent wheezing and asthma have been observed following severe RSV lower respiratory tract infection (LRTI), although a causal role for RSV in their inception remains contentious [6,7]. Incomplete protective immunity to RSV infection results in reinfections throughout life [8,9] and RSV is increasingly recognized as an important illness in elderly and immunocompromised individuals, with a disease burden similar to that of non-pandemic influenza A [10]. Despite its considerable medical importance, there are no effective and safe vaccines or RSV-specific therapeutics. Synagis, an anti-RSV F protein-specific humanized monoclonal antibody, which has demonstrated prophylactic efficacy against RSV, is restricted to infants considered at high risk of severe RSV disease. However, the majority of infants hospitalized with RSV do not fall into this category. Therefore, understanding how RSV causes disease in humans remains a major medical objective.

Severe RSV-induced disease in infants is commonly characterized by wheezing, hyperinflation, atelectasis, increased mucus secretion, cyanosis, tachypnea, and consolidation [2]. Histologic changes induced in the airways following RSV infection of infants were detailed in our recent comprehensive review [11]. Several characteristics of RSV-induced histopathology in the conducting airways were identified. For example, RSV infection was restricted to airway luminal ciliated epithelial cells and occasional non-ciliated cells. Non-contiguous or small clumps of infected cells were evident in large airways, whereas circumferential infection of apical cells was observed in some small airways. Infection was associated with a loss of ciliated cells, excess mucus production, and occlusion of some airways by sloughed epithelial cells, fibrin, mucus, and inflammatory cells, in particular macrophages and neutrophils. Infected airway epithelium cells demonstrated evidence of apoptosis, and occasional syncytia were also observed. Massive infiltration of immune cells, including neutrophils, macrophages, T cells, and, to a lesser extent, eosinophils, was common during severe RSV infection, and widespread destruction of airway epithelium was also evident.

Interestingly, close examination of immunohistochemically stained lung tissue from RSV-infected infants revealed vast areas of intact virus-infected epithelium [12]. This suggests that factors in addition to, or other than, RSV infection might be responsible for destruction of the airway epithelium, such as infiltrating inflammatory cells. Indeed, a number of proinflammatory cytokines and chemokines, including CXCL8, CXCL10, and CCL5, positively correlate with disease severity [13,14]. However, although RSV has been extensively studied in various models since its discovery [15], there remains a considerable paucity of knowledge concerning the mechanisms by which it causes disease in humans.

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Conventional models of RSV pathogenesis

The validity of any model of human infectious disease in elucidating mechanisms of disease, and thereby predictions for intervention strategies, depends entirely on how well it reproduces the hallmarks of the disease in humans. With the exception of palivizumab and RespiGam, which are antibodies used prophylactically to help prevent severe RSV disease in high-risk infants, models of RSV disease have so far failed to translate into effective interventions against RSV. The reasons for this failure are likely multifactorial and reflect the complex interactions of RSV with its human host.

Most studies on RSV pathogenesis to date were undertaken in either animal models or continuous cell lines. Although seminal insights into RSV–host interactions have emanated from animal models, these are only semi-permissive for RSV infection, with the possible exception of chimpanzees, and do not reflect the range of pathologies described in RSV-infected humans. However, recent advances with a mouse model of RSV infection are encouraging, in which some of the characteristic pathological changes evident in human lungs, such as epithelial desquamation, bronchiolitis, and induced airway mucin expression, were reproduced in BALB/c mice infected with RSV strain A2-20 [16]. It was recently reported that wild chimpanzees are susceptible to severe disease and death following RSV infection, although these reports were invariably associated with concurrent bacterial infections [17,18]. In any case, chimpanzees are a protected species and prohibitively expensive, and are essentially not amenable as an RSV infection model for the vast majority of laboratories worldwide. Immortalized airway-derived cell lines, such as HEp-2 and A549 cells, and primary epithelial cell monolayer cultures derived from human airways have also been extensively used to study RSV–host interactions [19–22]. However, they are poorly representative of the complexities of cell interactions in the human lung. Furthermore, primary human bronchial epithelial cells in monolayer cultures are considerably less susceptible to infection with recent RSV clinical isolates compared to the prototypic laboratory-adapted RSV A2 strain [23]. Therefore, the need for alternative models of RSV infection that more closely reflect RSV pathogenesis in humans is compelling.

WD-PAECs as models of RSV pathogenesis

Considerable research efforts over the past 30 years have culminated in a transwell system for culture of morphologically and physiologically authentic, well-differentiated primary airway epithelial cell cultures (WD-PAECs) *in vitro* [24–28]. In brief, PAECs are seeded onto a collagen-coated semi-permeable membrane that supports the cells and allows diffusion of nutrients from the growth medium (mimicking the lamina propria *in vivo*). Medium is placed in the apical and basolateral compartments of the transwell until cell confluence is reached. Once confluent, an air–liquid interface (ALI) is created by removing the medium on the apical compartment, which triggers differentiation of the cells. Some 3–4 weeks later, morphologically authentic WD-PAECs display a polarized, pseudostratified, multilayered epithelium comprising basal, ciliated, and goblet cells. However, although the gross morphology is very similar, differential cell counts suggest that there are some differences in the proportion of each cell type in WD-PAECs compared to airway epithelium *in vivo* [29,30]. The cultures closely mimic *in vivo* airway epithelium physiology in terms of cilia coverage and beating, mucus production, and intact tight junctions, as evidenced by robust transepithelial electrical resistance [26,28]. Furthermore, at the molecular level, comparative transcriptome profiles of WD-PAECs and *in vivo*-derived airway epithelium showed striking similarities in gene expression patterns, although differences were evident [29,30]. The cumulative data indicate that current WD-PAEC cultures are excellent surrogates for airway epithelium *in vivo*, although further improvements are possible. The primary airway epithelium cells were variously derived from postmortem lungs or from surgically resected bronchial or adenoid tissues, transplanted lungs from adults, or bronchial brushes in children [26,28,31].

Airway epithelium cells are the primary targets for many respiratory viruses, including RSV, and likely play central roles in the pathogenesis of associated diseases. Thus, WD-PAEC cultures provide exceptional opportunities to study respiratory virus–human airway epithelium interactions. A major advantage of these cultures is that the virus–epithelium interactions can be studied in the absence of immune cells and can thereby help in elucidating the roles of these interactions in pathogenesis. However, with respect to RSV, it is likely that these interactions function as important triggers, rather than as the main cause, of RSV pathogenesis, because RSV disease is widely considered to be immune-mediated [32,33]. Specifically in relation to RSV, a number of studies using WD-PAECs have been published since 1998, and these form the basis for this review.

RSV infection of WD-PAECs

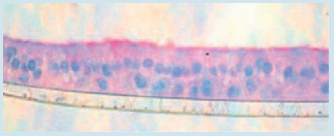
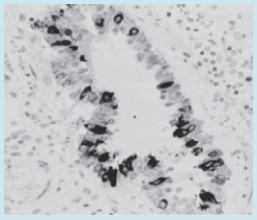
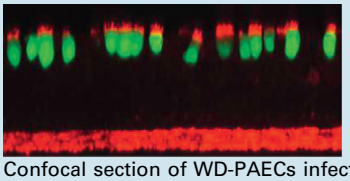
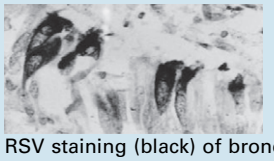
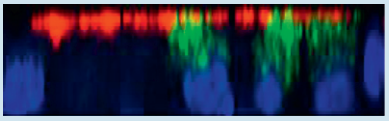
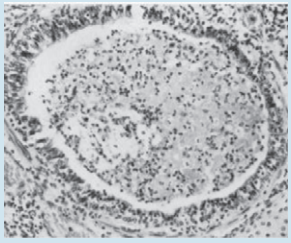
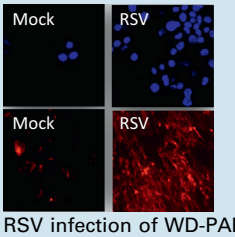
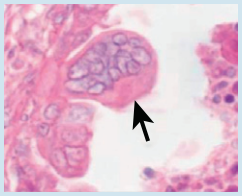
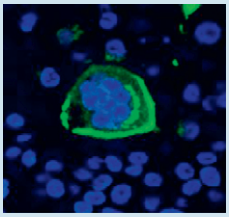
A striking feature of most studies using WD-PAECs is that RSV infection, even using a recent clinical isolate, causes little gross damage to the cultures [26,28,31,34]. Indeed, Wright *et al.* and Zhang *et al.* maintained RSV-infected cultures for 30 and 36 days, respectively, without obvious deterioration [26,35]. These data indicate that RSV infection is not directly responsible for destruction of the airway epithelium that is characteristic of RSV infection *in vivo*. Because large tracts of RSV-positive epithelium were also found to be intact *in vivo* [12,36], these *in vitro* observations concur with the current consensus that much of the destruction of airway epithelium following RSV infection is immune-mediated rather than directed by viral cytopathogenesis.

Consistent with histopathology from infant lungs, RSV infection of WD-PAECs was invariably restricted to apical ciliated and occasional non-ciliated cells [26,28,31,34]. There was no evidence of goblet cell infection. The apical restriction is unlikely to be due to intact tight junctions, because temporary chemical disruption of tight junctions did not result in basal cell infection [26]. Furthermore, mechanical damage to the cultures or basolateral inoculation resulted in only rare basal cell infection [26,31].

This suggests that basal cells are very refractory to RSV infection. Also consistent with histopathology observations [35], RSV infected non-contiguous cells or small clumps of cells in WD-PAECs, whereas not all ciliated cells were infected [26,28,31]. The reasons why many ciliated cells are refractory to infection remain to be elucidated. Syncytia formation is a hallmark of RSV infection in immortalized monolayer cells *in vitro*, and has occasionally been observed *in vivo*. In contrast to other reports, we routinely observed syncytia, albeit infrequently, in RSV-infected WD-PAECs [26,28,34]. The different origin of the cells (pediatric bronchial brushes vs enzymatically stripped epithelium from adult lung transplants) and/or our respective culturing conditions are possible explanations for these discrepancies.

Although gross damage was not evident following RSV infection of WD-PAECs, increased cell sloughing was observed [28,34]. Furthermore, most of the sloughed cells were apoptotic [28]. This is consistent with the contents of the mucus plugs that are hallmarks of RSV pathogenesis [36,37]. The sloughing was associated with increased ciliated cell numbers among sloughed cells and

Table 1. Comparison of human airway epithelium and WD-PAECs following RSV infection[a]

Hallmarks of RSV infection	In human airway epithelium	In WD-PAECs
Epithelium morphology	 Adult mucociliary pseudostratified bronchial epithelium supported by the submucosa [77]	 Well-differentiated primary airway epithelial cells after 21 days at air–liquid interface [39]
Location of RSV infected cells	 RSV immunostaining (black) in bronchial epithelial cells [36]; note the staining of apical noncontiguous or small clumps of cells	 Confocal section of WD-PAECs infected with GFP-expressing RSV (green) 24 h after infection; apical ciliated epithelial cells were stained using anti-keratin sulfate antibody (red) [26]
Cytoplasmic localization	 RSV staining (black) of bronchial epithelial cells; staining is strongest at the apical border of the cells [36]	 Anti-RSV F (green)-stained WD-PAECs 6 days post-infection; cilia are stained in red and nuclei in blue [28]. RSV staining was most intense near apical surfaces
Mucus and cell plug	 RSV infection forms plugs of exudate, mucus, and cell debris in a small bronchus [37]	 RSV infection of WD-PAECs causes apical cell sloughing (upper panels) and apical mucus secretion (lower panels) [28]
Syncytia formation	 Intrabronchiolar syncytia (arrow) are occasionally observed in RSV-infected infants [35]	 A syncytium (green) 6 days after RSV infection of WD-PAECs; RSV-F is stained in green, nuclei in blue [28]

[a]Images reprinted, with permission, from [26,28,35–37,39].

concomitant decreased ciliated cells in WD-PAEC cultures. Ciliostasis or reduction of cilia beat frequency has been observed in RSV-infected WD-PAECs [38]. A more recent study, however, demonstrated a dramatic reduction in the number of beating cells following RSV infection but no change in the beat frequency of cells that retained cilia [39]. Increased mucus production and goblet cell hyperplasia and/or metaplasia were also observed in RSV-infected WD-PAECs [28,39]. Thus, many of the hallmark cytopathological changes evident in RSV-infected airway epithelium *in vivo* are reproduced in RSV-infected WD-PAECs. Table 1 recapitulates the remarkable similarities between RSV-infected WD-PAECs and infant lung tissues.

In accordance with the apical restriction of infection, RSV shedding was polarized to the apical surface, with no evidence of virus present in the basolateral compartment [26,28,31]. Peak virus titers released from WD-PAECs ranged from approximately 10^5 to 10^7 infectious units/ml, depending on the publication. Similar RSV titers were detected in nasal and tracheal aspirates from hospitalized infants, although much lower titers were also reported [31,40,41]. The more extensive range of RSV titers evident in clinical samples may reflect aspirate sampling and handling procedures and/or the time of sampling following hospitalization. The kinetics of RSV shedding from WD-PAECs varied somewhat in different publications [26,28,31]. However, evidence presented by Wright *et al.* suggested that these discrepancies might be explained, to some degree at least, by different multiplicities of infection used during inoculation [35].

Some studies have demonstrated increased clinical severity associated with particular RSV strains [42–45]. However, the vast majority of laboratory experiments are undertaken with prototypic laboratory-adapted RSV strains (often A2 or Long), which may or may not reflect this strain-related pathogenesis. In attempts to address whether the RSV strain used is important to experimental outcomes, WD-PAECs were infected with RSV A2 in parallel with recent clinical isolates [28,31]. There were no differences in growth kinetics between the strains. However, RSV-induced cytopathogenesis tended to be more extensive following infection with the clinical isolate [28]. Thus, the choice of RSV strain used in WD-PAEC models may be important in elucidating molecular mechanisms of RSV pathogenesis.

Innate immune responses to RSV infection *in vivo* and in WD-PAECs

As well as providing a physical barrier to infectious agents, airway epithelial cells play an important role as a first line of immunological defense against invading pathogens. Indeed, RSV pathogenesis is driven, probably in large part, by infiltration of immune cells to the site of infection [12,36,46]. Several studies have investigated the infiltration of immune cells into the airway of children infected with RSV [12,36,46–51]. Neutrophils constituted the highest proportion of infiltrating cells in bronchoalveolar and nasal lavages and were also very common in autopsy tissues from RSV-infected infants [12,36,46–51]. They have also been detected between arterioles and airways [36]. Monocytes, macrophages, and lymphocytes were frequent in airways and lung parenchyma, and were also present in the lumenal debris, mixed with epithelial cells and mucus [36].

Infection of airway epithelium by RSV induces an innate immune response leading to secretion of cytokines and chemokines by the epithelium. The release of cytokines and chemokines and upregulation of adhesion molecules, such as ICAM-1 [39,52], mediate recruitment of leukocytes to the site of infection. CXCL8, CXCL10, CCL2, CCL3, CCL5, IL-1β, IL-6, IL-10, IL-18, and TNF-α are among a range of cytokines and chemokines that are increased in nasal aspirates, bronchoalveolar lavages, and blood from RSV-infected infants [13,51,53–59]. In particular, high levels of CXCL8 and CXCL10, major chemo-attractants for neutrophils, macrophages, and T cells, are hallmarks of RSV infection in infants [12,13,56,60]. Furthermore, the levels of some of these molecules correlated with disease severity. For example, CCL3 levels in nasopharyngeal secretions and IL-18^+ cells in nasal brushes were markedly increased in RSV bronchiolitis compared to mild RSV infections of the upper respiratory tract [53,58]. Interestingly, type I interferons (IFN-α/β), potent inducers of antiviral responses in infected and neighboring cells, were either absent or at very low levels in secretions from RSV-infected infants [61–63].

To determine whether RSV infection of WD-PAECs reflects *in vivo* innate immune responses to RSV, secretion levels of a number of cytokines and chemokines were quantified in apical and/or basolateral compartments of RSV- and mock-infected cultures. Consistent with the RSV-induced secretions *in vivo*, CXCL8, CXCL10, CCL5, and IL-6 secretion levels were increased in RSV-infected WD-PAECs derived from primary bronchial epithelial cells [28,38,64,65] and primary nasal epithelial cells [66]. Interestingly, very high basal levels of CXCL8 (30–50 ng/ml) were detected in non-infected cultures [28,38,65,66]. This might be an intrinsic feature of the model and might be induced by supplements added to the growth medium. Indeed, medium components such as retinoic acid, which is crucial for differentiation, have been shown to increase IL-8 secretion in human primary tracheobronchial cells [67]. Consistent with the lack of secretions *in vivo*, IFN-α/β was not detected following RSV infection of WD-PBECs [28]. Interestingly, Zhang *et al.* recently reported increased IFN-α/β secretions in their RSV–WD-PAEC model [34]. However, they used a biological assay to detect IFN-α/β, which does not preclude the possibility that the activity observed was due to type III IFNs rather than IFN-α/β. In this regard, using primary nasal epithelial-cell monolayer cultures, Okabayashi *et al.* recently demonstrated that type III IFNs were the predominant IFNs induced following infection with RSV [68]. However, a role for type III IFNs in RSV infection of WD-PAECs remains to be elucidated. Overall, it appears that a number of innate immune responses in RSV-infected WD-PAECs are consistent with those reported in secretions from RSV-infected infants. Table 2 recapitulates the considerable similarities in cytokine and chemokine secretions in aspirates from RSV-infected infant airways compared to WD-PAECs.

Table 2. Comparison of cytokine and chemokine levels in secretions from RSV-infected infants and WD-PAECs[a]

Cytokine or chemokine	RSV-infected infants	Refs	RSV-infected WD-PAECs	Refs
CXCL8	+++	[12–14,54,56,60,78]	+++	[28,38,64–66]
CXCL10	+++	[13,57]	+++	[28,64]
IL-6	++	[12,14,51,54,79,80]	++	[28,38,66]
CCL5	++	[13,14,55,56,60]	++	[28,38,64–66]
CCL2	++	[12,13,53,60]	++	[64]
CCL3	++	[13,14,60]	NA	
IL-1β	+/++	[12,54]	NA	
TNF-α	+	[12,51,79,80]	NA	
IL-10	+	[12,14,55]	NA	
IFN-α, IFN-β	–	[61–63]	–	[28]

[a]Symbols: +, <100 pg/ml; ++, 100–1000 pg/ml; +++, >1000 pg/ml; –, absence of analyte; NA, values not available for this particular analyte.

Concluding remarks and future directions

An increasing body of research has demonstrated that WD-PAECs are authentic surrogates of human airway epithelium *in vivo*. Similarly, RSV infection of WD-PAECs recapitulates many of the hallmarks of RSV cytopathogenesis in infants. At present, we are only beginning to exploit these models for RSV–human host interaction studies. Numerous questions may now be addressed regarding RSV cytopathogenesis in relevant human models, such as identification and use of RSV receptor(s), airway epithelial cell tropisms, and mechanisms of induction of innate immune responses. However, these cultures are technically difficult to work with and there is a limited window of opportunity in which they can be exploited. Improved culturing technologies and methodologies will considerably facilitate their exploitation. In this regard, human primary airway epithelial cells were successfully immortalized while retaining their ability to generate WD-PAECs [69–71]. This development may greatly expand our capacity to exploit these cultures.

Most of the research published to date has exploited tracheal and bronchial epithelium to generate WD-PAECs. However, RSV is a descending infection, commencing in the nasal epithelium and descending into the lungs. WD-PAECs derived from nasal epithelium are currently being generated, and it is expected that these will provide important insights into the first interactions of RSV with the human airway epithelium. They will also be of considerable interest in determining whether RSV interactions with nasal- and bronchial-derived WD-PAECs result in similar cytopathogenesis and innate immune responses. If so, nasal-derived WD-PAECs may act as surrogates for bronchial-derived WD-PAECs. This would be an important development because nasal brushes are considerably easier to procure than bronchial brushes and would open the possibility of generating WD-PAECs from cohorts of infants with defined clinical histories, such as those with histories of severe RSV disease and those who only ever suffered mild disease. The ability to group infants in this manner would facilitate novel studies on differential cytopathogenesis, innate immune responses, transcriptomes, and proteomes as a function of RSV disease severity. Such studies may provide important insights into why only a small minority of infants are hospitalized following RSV infection.

As discussed above, by mimicking the human airway epithelium in the absence of immune cells, WD-PAECs provide the opportunity to selectively study the role of human airway epithelium in RSV pathogenesis. However, it is well documented that the immune system plays a major role in RSV pathogenesis, with neutrophils and monocytes frequently observed in autopsies of children who died following RSV infection [11,32,36]. Numerous attempts have been undertaken to combine immune cells with airway epithelial cell monolayers in co-culture [72–75]. However, we identified only one study that investigated co-cultures of immune cells with WD-PAECs [76]. In this study, the authors demonstrated that influenza-virus-infected airway epithelium derived from smokers exhibited modified dendritic cell responses compared to non-smokers. This study demonstrates the potential to develop models to study interactions between virus-infected human airway epithelium and immune cells, either individually or as part of a complex mixture, as evident *in vivo*.

Because of the authenticity of these RSV–WD-PAEC models relative to RSV infection in infants, they offer a real opportunity to study RSV–human interactions at the cellular and molecular level. Thus, they are likely to yield significant breakthroughs in our understanding of RSV disease mechanisms in humans. Ultimately, they provide an exciting departure in translational research that may help to identify effective therapeutic strategies against RSV in humans, which have so far proved elusive.

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