Rhinovirus Delays Cell Repolarization in a Model of Injured/Regenerating Human Airway Epithelium

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Abstract

Rhinovirus (RV), which causes exacerbation in patients with chronic airway diseases, readily infects injured airway epithelium and has been reported to delay wound closure. In this study, we examined the effects of RV on cell repolarization and differentiation in a model of injured/regenerating airway epithelium (polarized, undifferentiated cells). RV causes only a transient barrier disruption in a model of normal (mucociliary-differentiated) airway epithelium. However, in the injury/regeneration model, RV prolongs barrier dysfunction and alters the differentiation of cells. The prolonged barrier dysfunction caused by RV was not a result of excessive cell death but was instead associated with epithelial-to-mesenchymal transition (EMT)-like features, such as reduced expression of the apicolateral junction and polarity complex proteins, E-cadherin, occludin, ZO-1, claudins 1 and 4, and Crumbs3 and increased expression of vimentin, a mesenchymal cell marker. The expression of Snail, a transcriptional repressor of tight and adherence junctions, was also up-regulated in RV-infected injured/regenerating airway epithelium, and inhibition of Snail reversed RV-induced EMT-like features. In addition, compared with sham-infected cells, the RV-infected injured/regenerating airway epithelium showed more goblet cells and fewer ciliated cells. Inhibition of epithelial growth factor receptor promoted repolarization of cells by inhibiting Snail and enhancing expression of E-cadherin, occludin, and Crumbs3 proteins, reduced

the number of goblet cells, and increased the number of ciliated cells. Together, these results suggest that RV not only disrupts barrier function, but also interferes with normal renewal of injured/regenerating airway epithelium by inducing EMT-like features and subsequent goblet cell hyperplasia.

Keywords: barrier function; epithelial-to-mesenchymal transition; Crumbs polarity complex; epithelial growth factor receptor; goblet cell hyperplasia

Clinical Relevance

To the best of our knowledge, this is the first study to demonstrate that rhinovirus delays cell repolarization in a model of injured/regenerating, but not normal, airway epithelium by inducing epithelial-to-mesenchymal transition-like features. Furthermore, rhinovirus reduces ciliated cell differentiation and promotes goblet-cell differentiation and mucin gene expression. These changes depended partially on persistent EGFR activation induced by rhinovirus. Such changes during regeneration can lead to airway epithelium with impaired barrier and mucociliary clearance functions.

Airway epithelium that lines the respiratory tract acts as a physical barrier between the external environment and the lung and plays an important role in maintaining

tissue homeostasis. Paracellular permeability of airway epithelium is maintained through the co-operation of two functionally distinct structural components:

tight and adherence junctions located in the apicolateral membranes. Tight junctions regulate the transport of solutes and ions across airway epithelium, and adherens

(Received in original form July 22, 2015; accepted in final form March 18, 2016)

This work was supported by National Institutes of Health grants HL897720, AT004793, and AT007620 (U.S.S.) and HL81420 (M.B.H.).

Author Contributions: A.N.F. and S.G. contributed to the design and conducting the experiments, data analysis, and interpretation; A.C., S.S.C., A.T.C., and B.L.U. contributed to conducting the experiments and data analysis; M.B.H. contributed to the data interpretation and critical evaluation of the manuscript; and U.S.S. contributed to the conception and design of the study, data interpretation, and manuscript preparation.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 55, Iss 4, pp 487–499, Oct 2016

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Originally Published in Press as DOI: 10.1165/rcmb.2015-0243OC on April 27, 2016

Internet address: www.atsjournals.org

junctions mediate cell-to-cell adhesion and promote polarization of cells and formation of tight junctions. In addition, airway epithelium expresses two polarity complexes: the Crumbs (CRB) complex and the partitioning defective complex (1). The CRB complex consists of an integral membrane protein, CRB3, and the scaffolding proteins, protein associated with Lin seven 1 and protein associated with Lin seven 1-associated tight junction protein. The CRB complex plays a critical role in the formation of tight junctions, cell polarization, and ciliogenesis in kidney epithelial cells (2, 3). Recently, we and others demonstrated that CRB3 may also contribute to cell polarization and differentiation and ciliogenesis in airway epithelial cells and is localized to the tips of the cilia in mucociliary-differentiated bronchial epithelium (4, 5). Disruption or abnormal expression of tight and adherence junction proteins, or CRB polarity complex proteins, may therefore significantly affect polarization of cells, formation of tight junctions, and cell differentiation. This, in turn, may lead to an abnormal airway epithelium with defective innate immunity including impaired barrier function and mucociliary clearance. Such changes could promote persistence and paracellular invasion of inhaled pathogens and could expose basolateral receptors, which are otherwise not accessible, to inhaled pathogens and allergens.

Rhinovirus (RV) is responsible for the majority of common colds and causes self-limiting upper-airway infections in normal subjects. In contrast, in patients with chronic airway diseases including chronic obstructive pulmonary disease (COPD), asthma, and cystic fibrosis, RV may cause prolonged and severe lower respiratory symptoms and, in the cases of COPD and cystic fibrosis, may lead to progression of lung disease (6–8). In those with COPD but not in healthy subjects, RV also promotes acquisition of secondary bacterial infections (8, 9). These observations indicate that RV, which infects and replicates primarily in airway epithelial cells (10), may impair innate immune functions of airway epithelium in subjects with chronic airway diseases, but the mechanisms are not well understood.

Previously, we demonstrated that RV reduces transepithelial resistance (TER) by dissociating ZO-1 and occludin from the apicolateral junction complex of airway epithelium without causing cell death

(11–13). RV has also been shown to delay wound closure in nonpolarized-undifferentiated bronchial epithelial cells (14). Furthermore, basal/undifferentiated cells, which are readily accessible after airway epithelial injury and during regeneration, are more susceptible to RV infection than are the suprabasal/differentiated cells (15). On the basis of the facts that RV readily infects basal epithelial cells (which are abundant in injured airway epithelium or epithelium showing basal cell hyperplasia) and causes delay in wound closure, we hypothesized that RV may interfere with the cell repolarization and normal renewal of injured/regenerating airway epithelial cells, leading to structurally and functionally abnormal airway epithelium.

In normal airway epithelium, cellular turnover is low, but after an injury, the epithelium is restored rapidly from the basal cell population. For instance, after an injury to airway epithelium, the surviving cytokeratin (Krt)⁵⁺, p63⁺ basal cells quickly spread to cover denuded areas and proliferate, polarize, and regenerate ciliated and secretory cells (16, 17). Therefore, polarized-undifferentiated basal cells can be considered as a model of injured/regenerating airway epithelium. As a first step toward understanding the effects of RV on airway epithelial repair, we used models of injured/regenerating (polarized-undifferentiated cells) and normal (mucociliary-differentiated cells) airway epithelium and monitored the long-term effects of RV on polarization and subsequent differentiation. We demonstrate, we believe for the first time, that although RV has no long-term effects on either barrier function or epithelial phenotype in a model of normal epithelium, it interferes with repolarization and normal differentiation of cells in a model of injured/regenerating airway epithelium.

Materials and Methods

Airway Epithelial Cell Cultures

Airway epithelial cells were isolated from tracheobronchial tissue of healthy nonsmokers, and the protocol for the collection of tracheobronchial segments was approved by the University of Michigan Investigational Review Board. Airway epithelial cells obtained from at least six donors were used. Primary airway epithelial

cells were cultured in Transwells at air–liquid interface as described previously, with some modifications (18, 19). Briefly, airway epithelial cells were cultured under submerged conditions in Bronchial Life media containing 25 nanograms per milliliter of epithelial growth factor (EGF) for 1 week. Cells were then shifted to differentiation media containing 0.5 nanograms per milliliter of EGF and maintained at air–liquid interface for 1 or 3 weeks to obtain a model of injured/regenerating airway epithelium (polarized-undifferentiated cell cultures), or a model of normal airway epithelium (polarized and mucociliary differentiated cell cultures), respectively (Figure 1A).

In some experiments, cells were transduced with pS1F1-H1-Snail or control shRNA lentivector during the first week of culturing.

RV and Infection

RV39 was propagated in H1 HeLa cells and partially purified and viral titer was determined as described previously (11). Less than a 100-kD fraction from purified RV preparation was used as a sham control.

Cultures were infected apically with RV at 1 MOI or at a similar volume of sham. Cells were incubated for 48 h at 33°C and then at 37°C for another 14 or 21 days. The basolateral medium was changed every other day during this period. In some experiments, RV-infected injured/regenerating airway epithelial cells were treated with 1 μ M erlotinib or dimethyl sulfoxide (DMSO) (vehicle control) for 10 or 17 days, starting from 4 days postinfection.

In selected experiments, the suprabasal/differentiated cell layer was removed from mucociliary-differentiated cell cultures by treating with accutase. The remaining layer of basal cells was allowed to polarize and was then used to assess the effect of RV on barrier function.

Determination of Viral Load

Infectious viral load in cell cultures was determined as described previously (11).

Measurement of TER and Paracellular Permeability

TER and the paracellular permeability of airway epithelial cell cultures were determined using Epithelial Voltohmmeter voltmeter and inulin, respectively, as described previously (11, 12, 20).

Figure 1. (A) Schematic representation of generating models of normal and injured/regenerating airway epithelium and rhinovirus (RV) infection and morphological features of airway epithelial models. At the end of the culturing period (i.e., after culturing at air-liquid interface for 1 or 3 wk), cell cultures were fixed and embedded in paraffin, and the cross-sections were stained with either H&E for general morphology or with PAS to detect goblet cells (B and C) by brightfield microscopy, or were subjected to immunostaining with antibody to Krt5 to detect basal cells by immunofluorescence (D and F). Arrows in C represent ciliated cells (left) and goblet cells (right). Identically grown cell cultures were fixed in paraformaldehyde and immunostained with antibody to occludin or E-cadherin and subjected to indirect immunofluorescence confocal microscopy (E and G). B to G are representative of cell cultures established from airway epithelial cells obtained from six donors. Scale bars: 50 μ m and 20 μ m. H&E, hematoxylin and eosin; PAS, periodic acid-Schiff.

Western Blot Analysis

Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors. An equal amount of protein was subjected

to Western blot analysis with antibodies to occludin, E-cadherin, CRB3, Snail, total or phosphor-GSK-3 β , or β -actin. Specific bands were quantified by

densitometry and expressed as fold change over β -actin.

Histology and Immunofluorescence Microscopy

Cross-sections of cell cultures were stained with hematoxylin and eosin or periodic acid Schiff and were subjected to light microscopy (18). Localization of occludin, E-cadherin, CRB3, cytokeratin 5, goblet cells, and cilia was determined by indirect immunofluorescence confocal microscopy, as described (11, 20).

Real-Time Polymerase Chain Reaction

The mRNA expression of occludin, E-cadherin, CRB3, vimentin, and MUC5AC was determined by using gene-specific primers and was presented as fold change over housekeeping genes, β -actin, or G3PDH.

Statistical Analysis

Statistical significance was assessed by unpaired Student *t* test (to compare two groups) or by analysis of variance with the Tukey-Kramer post hoc test (to compare three or more groups). A *P* value of ≤ 0.05 was considered statistically significant.

Results

Morphological Features of the Cell Culture Models

Models of injured/regenerating (polarized-undifferentiated) and normal (mucociliary-differentiated) airway epithelium were prepared as outlined in Figure 1A. To characterize these two models, cross-sections of normal and injured/regenerating airway epithelial cell cultures were prepared and stained with hematoxylin and eosin for assessing morphology and with periodic acid Schiff to detect goblet cells (Figure 1B). The model of injured/regenerating airway epithelium showed two to three layers of epithelial cells with no ciliated cells or goblet cells (Figure 1B). In addition, almost all the cells in this model were found to be Krt5⁺ (Figure 1D), a marker of basal cells, indicating enrichment of basal cells in this model. In contrast, the normal airway epithelial model showed two to three layers of epithelial cells with several ciliated and goblet cells (Figure 1C), and Krt5⁺ cells only in the basal layer of cells (Figure 1F). Both models showed distribution of occludin and E-cadherin around the cells,

indicating normal polarization and tight junction formation (Figures 1D and 1G).

RV Infection Impedes Repolarization of Airway Epithelial Cells in a Model of Injured/Regenerating Airway Epithelium

Models of injured/regenerating airway epithelium and normal airway epithelium were infected with RV at 1 MOI and monitored for TER for up to 14 days. Normal airway epithelial cell cultures infected with RV, but not with sham, showed a 30 to 40% drop in TER at 1 day postinfection, which returned to baseline by 7 days postinfection (Figure 2A). In contrast, compared with sham-treated cells, RV-infected injured/regenerating airway epithelial cell cultures showed a 60 to 70% reduction in TER at 1 day postinfection, which persisted for up to 14 days (Figure 2B). RV-induced reduction in TER was associated with increased paracellular permeability as assessed by diffusion of fluorescein isothiocyanate-labeled inulin from the apical to the basolateral chamber in both types of cell cultures (Figures 2C and 2D). At 14 days post-RV infection, injured/regenerating airway epithelial cell cultures also showed migration of apically added bacteria to the basolateral chamber (Figure 2E). In contrast, transmigration of bacteria was not observed in sham- or RV-infected normal cell cultures or in sham-infected injured/regenerating airway epithelial cell cultures. Despite such increases in paracellular permeability in RV-infected injured/regenerating airway epithelial cell cultures, no significant increase in lactose dehydrogenase (LDH) levels was observed (*see* Figure E1 in the online supplement). These results indicate that RV-induced prolonged barrier dysfunction in injured/regenerating epithelial cell cultures is not a result of cytotoxic effects.

The initial viral load was similar in both regenerating and normal epithelial cell cultures, but compared with injured/regenerating epithelial cell cultures, the viral load decreased rapidly in normal epithelial cell cultures (Figures E2A and E2B). Immunodetection of virus at 2 h postinfection again indicated a similar viral load in both models. However, although the virus was detected primarily on the apical surface of normal airway epithelial cell cultures, it was also detected deeper in an injured/regenerating airway epithelium model (Figure E2C). Even after decreasing the infecting viral dose by half,

injured/regenerating epithelial cell cultures showed persistent reduction in TER and the presence of an infectious virus for up to 7 days (Figure E3). These results indicate that injured/regenerating epithelial cell cultures may be defective in clearing viruses or restraining viral replication, and this may delay the repolarization of cells.

To determine whether the observed difference in the effects of RV on TER between the models of normal and injured/regenerating airway epithelium is caused by different culturing conditions, we assessed the effect of RV on injured mucociliary-differentiated normal airway epithelial cell cultures. The suprabasal layer of cells from normal epithelial cell cultures was removed by mild enzymatic treatment, and the remaining basal cells were allowed to polarize. The injured cell cultures showing TER of $\geq 400 \Omega \cdot \text{cm}^2$ were infected with sham or RV, and TER was monitored for up to 14 days. After RV infection, these injured cell cultures showed prolonged reduction in TER, similar to a model of injured/regenerating airway epithelium (Figure E4). These results not only indicate that the observed effects of RV in a model of injured/regenerating airway epithelium are not caused by culturing duration, but also that this model is suitable for assessing the long-term effects of RV.

RV Prolongs Barrier Dysfunction by Inducing Epithelial-to-Mesenchymal Transition-Like Features in a Model of Injured/Regenerating, but Not in Normal, Airway Epithelium

Normal or injured/regenerating airway epithelial cell cultures were infected with RV or sham and incubated for 14 or 21 days. The distribution of occludin and E-cadherin (representative proteins of tight and adherence junctions, respectively) and CRB3 (a representative protein of the CRB polarity complex) was assessed by indirect immunofluorescence confocal microscopy. Normal epithelial cell cultures infected with RV showed occludin and E-cadherin primarily in the periphery of cells similar to sham-infected cells (Figures 3A and 3C). As observed earlier (4), CRB3 was observed primarily in the tip of the cilia in both sham and RV-infected normal epithelial cell cultures (Figure 3E). Sham-infected injured/regenerating epithelial cell cultures showed distribution of occludin and E-cadherin (at 14 d postinfection) and CRB3 (at 21 d postinfection), similar to that

observed in sham-infected normal epithelial cell cultures (Figures 3B, 3D, and 3F). In contrast, RV-infected injured/regenerating epithelial cell cultures showed an overall reduction in the expression of all three proteins, with no intense staining for occludin and E-cadherin at the periphery of the cells (Figures 3B and 3D). These cultures also showed a reduction in cilia-associated CRB3 at 21 days postinfection (Figure 3F). Interestingly, we also observed fewer ciliated cells and a higher release of proinflammatory cytokines IL-6 and IL-8 in RV-infected, than in sham-infected, injured/regenerating airway epithelial cell cultures (Figures 3G–3I). At 21 days postinfection, there was no difference between sham- and RV-infected injured/regenerating epithelial cell cultures in the expression of ki67 mRNA (data not shown), a marker of proliferating basal epithelial cells, indicating that reduced ciliated cells are not a result of an increase in proliferating basal cells.

By Western blot analyses, RV-infected injured/regenerating epithelial cell cultures showed a significant reduction in the expression of occludin, E-cadherin, and CRB3 (Figures 4A–4D) compared with either sham-infected injured/regenerating epithelial cell cultures or RV- or sham-infected normal epithelial cell cultures. In addition, RV-infected injured/regenerating epithelial cell cultures showed attenuated mRNA expression of occludin, E-cadherin, and CRB3 (Figure 4E). These cultures also showed decreases in the expression of genes encoding other tight junction proteins such as zona occludin-1, claudin-1, and claudin-4 (Figure E5). RV-infected injured/regenerating epithelial cell cultures also showed higher mRNA expression of vimentin than did sham-infected cell cultures (Figure E6). Reduced expression of tight and adherence junction proteins and enhanced expression of vimentin are typical features of epithelial-to-mesenchymal transition (EMT). Together, these results imply that RV, which causes disruption of apicolateral junctions at early stages of infection in both normal and injured/regenerating epithelium, also interferes with the reformation of apicolateral junctions at later stages of infection by inducing EMT-like features, but only in injured/regenerating airway epithelial cell cultures.

Figure 2. RV infection causes persistent barrier dysfunction in injured/regenerating, but not in normal, airway epithelial cell cultures. Apical surface of normal (A, C, and E) or injured/regenerating (B and D) airway epithelial cell cultures was washed with 0.15% sodium bicarbonate and then with phosphate-buffered saline, infected apically with RV at 1 multiplicity of infection, or sham, and incubated up to 14 days. Transepithelial resistance (TER) (A and B) and paracellular permeability were measured by inulin flux from the apical to the basolateral chamber (C and D). Paracellular migration of bacteria from the apical to the basolateral chamber was determined at 14 days postinfection (E). Data represent mean $\pm$ SD (A–D) or median with range (E) calculated from experiments performed with airway epithelial cells obtained from three to six healthy nonsmokers. * $P \leq 0.05$, *t* test (A–D), different from respective sham controls; * $P \leq 0.05$, analysis of variance (E), different from other groups.

RV Enhances Expression of Snail Protein in a Model of Injured/Regenerating Airway Epithelium

EMT-like features are regulated by transcription repressors such as Snail, Slug, and Twist. Western blot analysis in our

initial studies revealed that airway epithelial cells express only Snail, but not Slug or Twist, in detectable amounts (data not shown). Snail has been demonstrated to negatively regulate proteins of the tight and adherence junctions and also CRB polarity complex (21–24). Therefore, we examined

whether decreased expression of E-cadherin, occludin, and CRB3 is associated with increased Snail expression in RV-infected injured/regenerating cell cultures. Compared with sham-infected cells, RV-infected cells showed significantly increased protein levels of Snail starting from Day 7, which persisted for up to 14 days postinfection (Figures 5A and 5B). However, there was no increase in Snail mRNA levels (Figure E7). Genetic inhibition of Snail expression partially blocked the RV-induced reduction in TER at 14 days postinfection, and this was associated with restoration of occludin and E-cadherin expression (Figures 5C and 5D), confirming the contribution of Snail to RV-induced EMT-like features.

Inactivation of GSK-3 β increases the accumulation of Snail protein levels without affecting transcription (25) and therefore, using Western blot analysis, we examined the levels of phospho-GSK-3 β (inactive form) in RV-infected cells. Compared with sham, RV infection increased GSK-3 β phosphorylation on Ser⁹ significantly at 7 days postinfection, which preceded increased abundance of Snail protein (Figures 5E and 5F). To examine whether GSK-3 β inhibition is sufficient to enhance Snail expression, injured/regenerating airway epithelial cell cultures were treated with GSK-3 β inhibitor XXVII for 48 h and Snail protein levels were assessed. Compared with vehicle (DMSO)-treated controls, GSK-3 β inhibitor-treated cultures showed a significant increase in Snail protein levels (Figures 5G and 5H).

RV-Induced GSK-3 β Phosphorylation Depends on Up-Regulation of Epithelial Growth Factor Receptor Activity in a Model of Injured/Regenerating Airway Epithelium

RV infection has been shown to phosphorylate and activate epithelial growth factor receptor (EGFR) in undifferentiated airway epithelial cells (26), and activation of growth factor receptors including EGFR can inactivate GSK-3 β (27). Therefore, we examined whether RV-induced EGFR activation is associated with phosphorylation of GSK-3 β by examining the phosphorylation of EGFR Tyr¹¹⁷³ (19). Compared with sham-, RV-infected cells showed a significant increase in EGFR phosphorylation as early as 4 days postinfection, which preceded GSK-3 β

Figure 3. RV alters the distribution of occludin, E-cadherin, Crumbs (CRB)3, and cell phenotype in a model of injured/regenerating airway epithelium. Normal (A, C, and E) or injured/regenerating (B, D, and F) epithelial cell cultures were infected with sham or RV and incubated for 14 or 21 days. Cell cultures at 14 days postinfection were immunostained with antibody to occludin (green, A and B), or E-cadherin (green, C and D). Cultures incubated for 21 days were immunostained with a combination of antibodies to CRB3 (red) and acetylated tubulin (to detect cilia, green) (E and F). Bound antibodies were detected by using appropriate secondary antibody conjugated with AlexaFluor 488 or 594. Cell cultures were counterstained with 4',6-diamidino-2-phenylindole to detect nuclei (blue) and were observed under a confocal microscope. Yellow in C and F represents co-localization of CRB3 with cilia. Images are representative of four individual experiments. The number of ciliated cells in a 0.1 mm² area was counted in 10 random fields per culture, and data are presented as mean $\pm$ SD (G). Cultures were shifted to a new receiver plate containing fresh medium at 20 days postinfection and incubated for another 24 h. Basolateral medium was used for detection of IL-6 and IL-8 by ELISA (H and I). Data represent mean $\pm$ SD calculated from six independent experiments. *P $\leq$ 0.05, *t* test, different from respective sham controls. Scale bars: 10 μ M and 20 μ M.

Figure 4. RV attenuates the expression of occludin, E-cadherin, and CRB3 in a model of injured/regenerating airway epithelial cell cultures. Normal and injured/regenerating airway epithelial cell cultures were infected with sham or RV as above and incubated for 14 days. Cells were lysed, and the cell lysates were subjected to Western blot analysis with antibody to occludin, E-cadherin, CRB3, or β -actin (A). The band intensities in Western blots were quantified and expressed as fold change over β -actin (B–D). Images in A are representatives of three or four independent experiments. From identical experiments, total RNA was extracted and used to determine the mRNA expression of occludin, E-cadherin, and CRB3, and data are expressed as fold change over β -actin (Δ Ct). Data (B–E) represent mean $\pm$ SD calculated from three or four independent experiments. *Analysis of variance, different from sham-infected injured/regenerating epithelial cell cultures; # and § analysis of variance, different from RV- or sham-infected normal epithelial cell cultures, respectively.

phosphorylation (Figures 6A and 6B). Although EGFR phosphorylation in RV-infected cells decreased with time, it remained significantly higher compared with that in sham-infected cells for up to 14 days postinfection. RV-infected cells treated with erlotinib, a specific pharmacological inhibitor of EGFR (28), for 10 days starting

4 days after infection, showed a reduction in phosphorylation of GSK-3 β and Snail protein abundance (Figure 6C) and an increase in the expression of occludin, E-cadherin, and CRB3, compared with vehicle group (Figure 6D). These results indicate that EGFR activation induced by RV may be responsible for GSK-3 β phosphorylation

and subsequent reductions in the expression of apical junction complex proteins.

Inhibition of RV-Induced EGFR Activation Promotes Repolarization and Differentiation of Cells

RV- or sham-infected cells were treated with DMSO or erlotinib for 10 or 17 days starting

Figure 5. RV increases protein levels of Snail and phosphorylation of GSK-3 β . Injured/regenerating airway epithelial cell cultures were infected with sham or RV and incubated for up to 14 days. Total proteins isolated at 4, 7, 10, or 14 days were subjected to Western blot analysis with antibody to Snail or β -actin (A), and the band intensities were quantified and expressed as fold change over β -actin (B). Cell cultures transduced with either control or Snail small hairpin RNA (shRNA) were infected with sham or RV and incubated for 14 days. TER (C) and expression of Snail, occludin, E-cadherin, and Crumbs3 was determined (D). Total proteins from sham or RV-infected nontransduced injured/regenerating airway epithelial cell cultures were subjected to Western blot analysis with antibody to phospho-GSK-3 β or total GSK-3 β (E) and expressed as the ratio of phospho-GSK-3 β or total GSK-3 β (F). Injured/regenerating airway epithelial cell cultures were treated with DMSO (vehicle control) or GSK-3 β inhibitor XXVII. After 48 h, the cells were lysed and subjected to Western blot analysis with anti-Snail antibody (G), and band intensities were quantified and expressed as fold change over β -actin (H). Western blots are representative of three independent experiments. Data in B, C, F, and H represent mean $\pm$ SD calculated from three independent experiments. * $P \leq 0.05$, analysis of variance, different from respective sham (B, C, and F). # $P \leq 0.05$, analysis of variance, different from control shRNA (F); * $P \leq 0.05$, t test, different from vehicle control (H). GSK-3 β , glycogen synthase kinase 3 β ; p-GSK, phospho-GSK.

from 4 days postinfection, and TER was determined. As observed earlier, compared with sham-infected cells, RV-infected cells showed persistent reductions in TER (Figure 7A). Treatment with erlotinib for 10 days restored the TER of RV-infected cells. This was accompanied by increased expression and distribution of both occludin and E-cadherin to the periphery of cells, closely resembling sham-infected cells (Figure 7B). Cultures treated with erlotinib for 17 days not only showed an increased association of CRB3 with cilia (Figure 7C), but also an increased number of ciliated cells and a decreased number of goblet cells and mucin gene expression compared with DMSO-treated cells (Figures 7D and 7F). In addition, treatment with erlotinib reduced the release of IL-8 and IL-6 by these cultures (Figure E8). However, there was no significant difference in RV load between erlotinib- and vehicle-treated cell cultures at 7 days postinfection (Figure E9). As observed earlier, no infectious virus could be detected 10 days postinfection, irrespective of the treatment.

Together, these results imply that EGFR activation may contribute to RV-induced EMT-like features and altered cell differentiation in a model of injured/regenerating airway epithelium.

Discussion

This study highlights one of the mechanisms by which RV may interfere with the regeneration of airway epithelium after injury. In this study, we used mucociliary-differentiated cell cultures and polarized-undifferentiated cell cultures as models of normal and injured/regenerating airway epithelium, respectively. We demonstrate, we believe for the first time, that RV prevents the repolarization of cells, reduces the number of ciliated cells, and promotes goblet cell hyperplasia in a model of injured/regenerating airway epithelium, but not in a model of normal airway epithelium. Another novel finding is that RV prolongs barrier dysfunction in injured/regenerating epithelium by inducing EMT-like features, such as reduced expression of E-cadherin and occludin and enhanced expression of vimentin and Snail. RV also reduced the expression of CRB3, a component of the CRB polarity complex, which has been shown to play a role in cell polarization and differentiation and

ciliogenesis (3–5). These effects depended partially on sustained EGFR activation. Such changes in airway epithelial structure and function may impair the innate immunity of regenerated airway epithelium.

We and others have demonstrated previously that RV causes disruption of barrier function in both normal mucociliary-differentiated airway epithelial cell cultures and in polarized-undifferentiated cell cultures (11, 12, 20, 29). However, we believe this is the first study to examine the effects of RV on repolarization and the subsequent differentiation of cells. Here, we show that RV, in addition to causing barrier disruption at early stages of infection, inhibits the repolarization of cells, causing prolonged barrier dysfunction, in a model of injured/regenerating airway epithelium. This is caused by defective wound closure or cell death, because RV does not cause the excessive cytotoxic effects that create wounds in the cultures. In addition, in a wound closure assay, RV-infected cells showed a similar rate of wound closing as did sham-infected cells (Figure E10). Interestingly, we found that RV causes prolonged barrier dysfunction by inducing EMT-like features such as reduced expression of occludin, E-cadherin, and CRB3 and increased expression of vimentin and Snail (30). Although induction of EMT-like changes after injury is a necessary first step during repair, persistence of such changes as observed in the RV-infected injured/regenerating airway epithelial model may prevent repolarization and alter subsequent cell differentiation.

During EMT, the expression and activation of Snail has been shown to be up-regulated in epithelial cells (31). Snail is a transcriptional repressor and inhibits the expression of tight junction proteins including occludin, ZO-1, claudin-1 (22, 24), and the adherence junction protein E-cadherin (21), thereby preventing the formation of the apicolateral junctional complexes that contribute to barrier function. Snail also represses the expression of CRB3 and prevents polarization and ciliogenesis (3, 4). We found that RV increases Snail protein levels without altering mRNA levels, indicating that RV regulates Snail at the post-translational level. Consistent with this notion, RV was found to increase the phosphorylated (inactivated) form of GSK-3 β at 7 days postinfection, which precedes increases in

Snail protein levels. Because GSK-3 β phosphorylates and targets Snail to proteasomal degradation (32), it is likely that RV promotes accumulation and subsequent activation of Snail by inactivating GSK-3 β in injured/regenerating epithelial cells. Inhibition of RV-induced EGFR blocked GSK-3 β phosphorylation and Snail accumulation, indicating a role for EGFR in this process.

Another major finding of this study is that RV altered the differentiation of cells in the injured/regenerating airway epithelial model, an effect which was again dependent partially on EGFR activation. Although EGFR plays a pivotal role in the repair of injured airway epithelium (33), sustained EGFR activation may impede the recovery of barrier function in injured airway epithelium (34), adversely affect cell differentiation (35, 36), and induce proinflammatory phenotype (19). Persistent activation of EGFR in mucociliary-differentiated cell cultures by treatment with reactive oxygen species has been demonstrated to reduce ciliated cells and to induce goblet-cell metaplasia (35). Chronic EGFR activation by Sendai virus induces goblet-cell metaplasia in well-differentiated airway epithelial cells by promoting survival and transdifferentiation of ciliated cells to goblet cells (36). EGFR activation in basal airway epithelial cells reduces the expression of genes involved in ciliogenesis and the secretory phenotype, shifting cells toward squamous and EMT-like phenotypes (37). In this study, we found that RV, by activating EGFR, inhibits the expression of E-cadherin and CRB3, each of which regulates the polarization of cells, the event that dictates subsequent cell differentiation (3–5). However, it should be noted that inhibition of EGFR activity had no effect on viral load, demonstrating that inhibition of EGFR does not suppress differentiation by increasing the susceptibility to viral infection.

RV infection has been shown to induce EGFR activation in nonpolarized airway epithelial cells by activation of matrix metalloproteinases, which subsequently release EGF ligands from the cell surface (26). However, in polarized epithelial cells, E-cadherin interacts and retains EGFR at the basolateral surface, thereby preventing its activation by apically released EGFR ligands (38). Therefore, endogenous EGFR activation in polarized

Figure 6. RV-induced epithelial growth factor receptor (EGFR) activation is responsible for changes in the phosphorylation of GSK-3 β and snail accumulation and attenuation of occludin, E-cadherin, and CRB3 expression. Sham- or RV-infected injured/regenerating airway epithelial cell cultures were incubated for up to 14 days. Cell lysates prepared at 2, 4, 7, 10, and 14 days postinfection were subjected to Western blot analysis with antibody to p-EGFR or total EGFR (A), and the band intensities were quantified and expressed as a ratio of phospho- to total EGFR (B). Injured/regenerating airway epithelial cell cultures were infected with sham or RV and treated with EGFR inhibitor, erlotinib, or DMSO (vehicle control) for 14 days starting from 4 days postinfection. Total proteins were isolated and subjected to Western blot analysis with antibodies to p- or total GSK-3 β , Snail, occludin, E-cadherin, CRB3, or β -actin (C and D). Western blots are representative of at least three independent experiments. Data in B represents mean $\pm$ SD calculated from four independent experiments. * $P \leq 0.05$, analysis of variance, different from respective sham. p-EGFR, phospho-EGFR.

epithelial cells not only requires ligand availability but also translocation of EGFR from the basolateral to the apical surface (34). One of the mechanisms that can promote EGFR translocation to the apical surface is destabilization of adherence junctions, which disrupts the interaction of EGFR with E-cadherin. Our ongoing studies indicate increased expression of amphiregulin, an EGFR ligand, as well as dissociation of E-cadherin from the adherence junctions in a model of injured/regenerating epithelium but not in a model of normal airway epithelium at 48 h postinfection (data not shown). On the basis of these recent findings, we speculate that in the injured/regenerating epithelium, RV may promote EGFR translocation to the apical surface and subsequent activation by amphiregulin. At present, we are conducting additional studies to confirm

these findings, and this will be a subject for future publication.

Compared with injured/regenerating airway epithelial cell cultures, normal cell cultures were less sensitive to RV-induced changes in barrier function or phenotypic changes. This may be because of their differential susceptibility to RV infection. Previously, basal undifferentiated airway epithelial cells have been shown to be more susceptible to RV than are suprabasal mucociliary-differentiated cells (15). This difference was attributed to higher expression of intercellular adhesion molecule (ICAM)-1, a receptor for major group RV in basal epithelial cells. Because our model of injured/regenerating airway epithelium was enriched in basal epithelial cells, we speculated that the observed RV-induced changes in cell repolarization and the subsequent differentiation may be a result of greater expression of ICAM-1 on

these cells. Interestingly, there was no difference in the apical expression of ICAM-1 between injured/regenerating and mucociliary-differentiated cell cultures (data not shown). This may be because the cell cultures used in this study are polarized, as opposed to the nonpolarized basal epithelial cells used in the previous study. In addition, there was no difference in the initial viral load (i.e., 2 h post-RV infection) between the normal and injured/regenerating airway epithelial cell cultures. These observations imply that RV-induced changes in barrier function and phenotype in the injured/regenerating airway epithelial cell cultures (i.e., endocytosis, viral replication, or defective viral clearance) are initiated after the interaction of RV with ICAM-1. We found that, consistent with this notion, whereas mucociliary differentiated cultures rapidly cleared infectious virus,

Figure 7. Erlotinib, an EGFR inhibitor, partially reverses RV-induced effects on TER and increases the number of ciliated cells in injured/regenerating airway epithelial cell cultures. Sham- or RV-infected injured/regenerating epithelial cell cultures were incubated with either DMSO or 1 μM erlotinib for 10 or 17 days starting from 4 days postinfection. TER was determined as described in Figure 2A (A). After 10 days of treatment with erlotinib, cell cultures were fixed and immunostained with antibody to occludin or E-cadherin to determine the distribution of occludin and E-cadherin (B). Scale bars: 20 μM . Cell cultures fixed after 17 days of treatment with erlotinib were immunostained with a mixture of antibodies to acetylated tubulin (to detect cilia, green) and CRB3 (red) (C) and antibodies to acetylated tubulin (green) and MUC5AC (to detect goblet cells, red) (D). Scale bars: 10 μM . The density of ciliated and goblet cells was determined by counting the number of ciliated cells/0.1 mm^2 in 10 random fields (E). At 24 h postinfection, total RNA was isolated, and the expression of MUC5AC mRNA was determined by quantitative polymerase chain reaction (F). Data in A, E, and F represent mean $\pm$ SD calculated from three independent experiments. * $P \leq 0.05$, t test, different from respective sham; # $P \leq 0.05$, t test, different from RV-infected DMSO-treated cell cultures. Images in B, C, and D are representative of three to four experiments. MUC5AC, mucin 5AC.

injured/regenerating epithelial cell cultures showed virus for up to 7 days postinfection. Moreover, we detected more intracellular virus in injured/regenerating epithelial cell cultures, indicating increased endocytosis or viral replication in these cell cultures. One of the plausible mechanisms underlying enhanced viral replication and/or defective viral clearance in injured/regenerating epithelial cell cultures may be a lack of nitric oxide production in these cell cultures. Nitric oxide, which enhances viral clearance and limits RV-stimulated proinflammatory cytokines (39, 40), is produced primarily by ciliated cells in the airway epithelium (41), and these cells are absent in injured/regenerating epithelium. Finally, undifferentiated polarized cells also showed higher expression of EGFR than did mucociliary-differentiated cells (37, 42). Therefore, it is likely that RV, which is not cleared effectively in

injured/regenerating airway epithelial cell cultures, may activate the EGFR that is already highly expressed in injured/regenerating cells, thereby prolonging barrier dysfunction and promoting abnormal differentiation.

Conclusions

On the basis of these observations, we speculate that in chronic airway diseases including COPD, asthma, and cystic fibrosis, the airways of which show both an increased basal cell population (43–45) and enhanced expression of EGFR (19, 46–49), RV may alter the structure and function of airway epithelium and further impair innate defense functions, including barrier function and mucociliary clearance mechanisms. In turn, this may increase the susceptibility to secondary infection by facilitating the crossing of the mucosal barrier by pathogens and their interaction with basolateral receptors, thus altering the

cytokine and chemokine responses. In addition, such changes in innate defense mechanisms could enhance chemokine responses to other environmental factors, leading to heightened inflammation and progression of lung disease in these patients. Even though EGFR inhibition reverses RV-induced changes in the injured/regenerating airway epithelium, limiting viral replication or enhancing viral clearance may be a better option for alleviating RV-induced alterations in regenerating airway epithelium, because inhibition of EGFR has been shown to induce adverse events in patients with COPD. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank the National Disease Research Interchange, Philadelphia, PA, for providing trachea from healthy nonsmokers. They also thank Brenton Kinker for editing the manuscript.

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