Rhinovirus infection induces extracellular matrix protein deposition in asthmatic and nonasthmatic airway smooth muscle cells

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Asthma is a chronic inflammatory disease of the airways that is characterized by airway remodeling. The etiology of remodeling remains to be fully elucidated; however, it is likely to be the result of an aberrant repair cycle in response to inflammation. The histological characteristics of airway remodeling include increased extracellular matrix (ECM) deposition and increased airway smooth muscle (ASM) bulk. Remodeling is important, as it correlates with disease severity (7), contributes to fixed airway obstruction, and may drive the accelerated loss of lung function seen in asthma.

The ECM consists of multiple protein macromolecules, which, in addition to providing structural support, can mediate cellular functions. Several ECM proteins have been shown to be altered in asthma; for example, fibronectin, collagen I, II, and V, tenascin, hyaluronan, versican, laminin-α2/β2, lumican, and biglycan are increased (2, 23, 24, 34, 35), while collagen IV and elastin are decreased (4). In vitro studies have shown that the ECM modulates cell proliferation and migration and the release of inflammatory mediators and growth factors (11, 19). Furthermore, in vivo studies have shown that ECM proteins influence airway hyperresponsiveness, lung function, and airway responses to deep inspiration (38), thus demonstrating the impact of the ECM environment on ASM function.

Viral respiratory infections are the most common cause of childhood wheezing (1, 16) and asthma exacerbations in children and adults (21, 27). Rhinovirus (RV) is the most common virus type identified in the airways of asthmatic subjects during episodes of asthma exacerbations (8). RV-induced wheezing during infancy in children with a genetic predisposition for developing allergic respiratory disease is associated with increased risk for developing asthma (14, 25).

Viruses activate the innate immune response through activation of Toll-like receptor (TLR) 3 (TLR3) and TLR7/8. ASM cells express TLR3 and TLR7/8 under basal conditions, and TLR3 and TLR7 expression dramatically increased when ASM cells were stimulated with TNFα (39). The receptor agonists imiquimod and polyinosinic:polycytidylic acid (poly I:C) are synthetic analogs of single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA). Poly I:C is known to activate TLR3, retinoic acid-inducible protein I, and melanoma-differentiated-associated gene 5 (22), while imiquimod is an agonist for TLR7/8 (26).

As RVs are known to induce airway inflammation and growth factor release (33, 41), it is also possible that they may induce ECM production. While the principal site of RV infection in the lower airways is the epithelium, several studies have suggested that submucosal infection occurs (29) even in the absence of any clinical symptoms (15).

Few studies have investigated the role of RV infection on airway remodeling, and no studies have examined ECM production from RV-infected human ASM cells. We hypothesize that RV infection induces remodeling of the airways through ECM deposition and that this modified ECM will affect cell proliferation and migration, further contributing to the features of airway remodeling.
METHODS

Cell culture. Primary human (ASM) cells were obtained from lung specimens in accordance with the Human Ethics Committees of the University of Sydney and the South West Sydney Area Health Service. ASM cells from nonasthmatic (56.8 ± 14.6 yr old) and asthmatic (35.4 ± 14.3 yr) subjects were isolated from bronchial tissue. Subject details are shown in Supplemental Table S1 (see Supplemental Material for this article, available online at the Journal website). Human ASM bundles were dissected free from surrounding tissue and grown as explants in DMEM with 10% FBS and 20 U/ml penicillin, 20 μg/ml streptomycin, and 2.5 μg/ml amphotericin B, as previously described. ASM characterization was confirmed by means of light microscopy and immunohistochemistry for smooth muscle α-actin and calponin expression. ASM cells were used between passages 4 and 8 and were seeded at a density of 1 × 10^5 cells/cm² and allowed to grow until fully confluent (7 days) prior to treatment.

RV propagation, titration, and purification. Stocks of human RV-2 and RV-16 (American Type Culture Collection) were amplified by growth in Ohio HeLa cells, as previously described (30). Virus was purified using a 100,000-molecular-weight cutoff filter, as previously described (3).

ECM protein ELISA. ASM cells were seeded in 96-well plates and treated as described above for 48 h. ECM free of cells was prepared by treatment with sterile hypotonic ammonium hydroxide (0.016 M). The exposed ECM proteins were measured by ELISA, as described elsewhere (17), using the following antibodies: chondroitin sulfate (mouse anti-human; Sigma), collagen I (rabbit polyclonal; Abcam, Cambridge, UK), collagen IV (sheep anti-mouse, biotin-conjugated; Chemicon, Temecula, CA), collagen V (rabbit polyclonal to collagen V, 2 μg/ml; Abcam), elastin (clone BA-4 monoclonal; Sigma), perlecain (clone 7B5, mouse anti-perlecain, 2 μg/ml; Zymed Laboratories, Carlsbad, CA), VEGF-A (biotinylated anti-human VEGF antibody, 300 ng/ml; R & D Systems, Minneapolis, MN), versican (rat anti-human IgG; R & D Systems), tenascin (mouse monoclonal anti-human; Sigma), purified mouse IgG1 isotype control (clone MOPC-31C, 2 μg/ml; Becton Dickinson Pharmingen, San Jose, CA), rabbit isotype (2 μg/ml; Dako), sheep anti-mouse Ig affinity-isolated bontin-conjugated (Chemicon), anti-mouse IgG-peroxidase (HRP)-polyclonal rabbit anti-mouse; Dako), and streptavidin-HRP (R & D Systems).

RV RNA extraction and quantification. RV RNA was extracted using a total RNA isolation kit (Macherey-Nagel, Bethlehem, PA) with slight modifications to the manufacturer’s instructions. Briefly, 10 ml of RV stock were added to 10 ml of cell lysis buffer and filtered through six lysate filters. An equal volume of 70% ethanol was added to the filtrate and spun in six RNA binding membrane columns. Each washing of the membrane was carried out according to the manufacturer’s instructions. RNA was eluted into a total volume of 240 μl. RNA concentration was measured using a spectrophotometer (NanoDrop ND-1000, ThermoScientific, Wilmington, DE).

Quantitative real-time PCR. Total RNA was extracted from the cells at 6 h using a total RNA isolation kit (Macherey-Nagel) and reverse-transcribed using random primers (New England Biolabs, Ipswich, MA). Real-time PCR was performed as previously described (6) using commercially developed primers [His00365058_m1 (FN) and His01785336_m1 (perlec)] Applied Biosystems, Foster City, CA]. mRNA expression was normalized against that obtained for 18S rRNA and quantified using the cycle threshold (ΔΔCT) method.

RV infection and RV RNA, poly I:C, and imiquimod treatment. On the day of infection, fresh medium was added containing RV at multiplicity of infection (MOI) of 0.1 and 1. ASM cells were infected in DMEM supplemented with 2% FBS. Cells were incubated at 37°C with shaking every 15 min for 1 h; then fresh medium without virus was added. Imiquimod at 0.3 and 3 μg/ml (InvivoGen, San Diego, CA), poly I:C at 0.1 and 1 μg/ml (Sigma), and RV-16 RNA (extracted from RV-16 stock) at 0.1 and 1 μg/ml were diluted in 10% FBS and used to treat the cells for 48 h.

ECM reseeding. ECM plates were prepared from cells grown in 96-well plates with or without viral infection. At 48 h after virus exposure, cells were lysed using ammonium hydroxide (0.016 M), washed five times with PBS under sterile conditions, and stored at −20°C. The plates were then thawed and washed with DMEM before the plates were reseeded with cells. ASM cells were reseeded with 1 × 10^4 cells/cm² for 5 days in 5% FBS in DMEM before measurement of proliferation, proteins in the supernatant, and matrix proteins.

Migration assay. Matrix migration was conducted using 8-μm Transwell inserts (Becton Dickinson Pharmingen). Briefly, ASM cells were seeded with 1 × 10^4 cells/cm² in Transwell inserts for 7 days followed by RV infection for 2 days at MOI of 0.1 and 1. At day 2 of infection, the cells were lysed with 0.016 M ammonium hydroxide as described for cell reseeding. The Transwell inserts were frozen at −20°C in sterile PBS before use in the migration assay. RV-naïve cells used for migration were seeded in culture at 1 × 10^5 cells/cm² for 4 days and quiesced with DMEM supplemented with 0.1% BSA for 3 days. On the day of migration, cells were trypsinized, washed three times with PBS, and resuspended into DMEM at 2.5 × 10^5 cells/ml, and 100 μl were placed in the washed (4 times in sterile PBS) Transwell insert (with a prelaid ECM generated from RV-treated ASM cells) for migration at 37°C with 5% CO₂. Complete growth medium was used as a chemoattractant, or DMEM without any supplements was used as a control. After 4 h, the Transwell insert was washed twice with warm sterile PBS and placed in a new 24-well plate containing 0.6 ml of cell dissociation solution (Trevigen, Gaithersburg, MD) and 1.67 μg/ml calcein-AM (Invitrogen, Carlsbad, CA) to both label and remove cells that had migrated through the Transwell insert. Quantification was carried out by measurement of the fluorescence [485-nm excitation and 520-nm emission on the SpectraMax M2 plate reader (Molecular Devices, Camberwell, Australia)].

Proliferation assay. Proliferation was assessed by means of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] assay on day 5, as previously described (10). Absorbance was measured at a test wavelength of 570 nm and a reference wavelength of 655 nm.

Analysis of data. For experiments in which measurements were compared between a constitutive and an experimental response observed in cells from the same donor, repeated-measures ANOVA with Dunnett’s multiple comparisons was performed on the results for all ELISAs and RT-PCR assays where appropriate. Post hoc tests were carried out only on data tables that were shown to be significantly different by ANOVA. In all cases, P ≤ 0.05 was considered significant. For matrix migration experiments, each matrix was normalized to its respective matrix equivalent with cells in the absence of chemoattractant.

RESULTS

RV increases deposition of ECM from nonasthmatic ASM cells. To determine whether RV infection plays a role in airway remodeling, ASM cells from nonasthmatic subjects were infected with RV, and the ECM proteins deposited in the matrix surrounding these cells were measured using ECM ELISAs. A panel of ECM proteins, including collagen I, collagen IV, collagen V, chondroitin sulfate, elastin, fibronectin, perlecain, tenascin, versican, and elastin, was measured; we found that infection of ASM cells with crude RV-16 inoculum increased only fibronectin (41%) and perlecain (78%) at MOI 1 (Fig. 1). To confirm that the induction of ECM was due to RV infection and not the result of nonviral components in the viral inoculum, cells were also infected with purified RV-16. This also increased fibronectin (26%) and perlecain (42%) deposition at MOI 1 compared with uninfected cells. To investigate whether this response was receptor-specific, purified minor
group RV, which infects cells via a different receptor, RV-2, was also used to infect the cells. Purified RV-2 increased fibronectin (30% at MOI 0.1) and perlecan (45% and 49% at MOI 0.1 and 1, respectively), demonstrating that the responses were not receptor-specific (Fig. 1). We also assessed ECM protein mRNA production to establish if increased protein production was accompanied by increased mRNA production. Real-time PCR showed increased expression of fibronectin and perlecan mRNA by purified RV-16 and RV-2 (Fig. 2). At the MOI of RV used in this study, no cytotoxicity, as assessed by the MTT assay, was found (see Supplemental Fig. D3).

TLR agonists also induce ECM deposition. To determine the mechanism by which RV induces ECM deposition, the TLR agonists poly I:C and imiquimod were used. We found that the TLR3 agonist poly I:C increased the deposition of fibronectin (20% at 0.1 µg/ml) and perlecan (37% at 0.1 and 1 µg/ml). The TLR7/8 agonist imiquimod also increased the deposition of fibronectin (32% and 21% at 0.3 and 3 µg/ml, respectively) and perlecan (39% and 31% at 0.3 and 3 µg/ml, respectively). Pure RV-16 RNA also increased perlecan (35% at 0.1 µg/ml) deposition only (Fig. 3).

RV induces different ECM deposition in asthmatic ASM cells. We obtained ASM cells from asthmatic subjects and infected the cells to assess whether RV-induced ECM deposition is the same as that in nonasthmatic cells by measuring the same panel of ECM proteins. In accordance with the findings in nonasthmatic ASM cells, crude RV-16 inoculum induced fibronectin (74% at MOI 1), but, in stark contrast, perlecan was not induced, while the deposition of collagen IV was increased (13% at MOI 1; Fig. 4). We further investigated the induction of collagen IV and found that poly I:C (17% and 41% at 0.1 and 1 µg/ml, respectively), imiquimod (67% and 93% at 0.3 and 3 µg/ml, respectively), and RV-16 RNA (100% and 114% at 0.1 and 1 µg/ml, respectively) induced the deposition of collagen IV (P < 0.01, n = 4; data not shown).

RV-modulated ECM increases ASM migration and decreases proliferation of ASM. We have been able to show that RV increases the deposition of ECM proteins, and the profile of increased ECM proteins is different between ASM cells derived from nonasthmatic and asthmatic subjects. To determine the functional significance of RV-induced ECM deposition, virus-naive nonasthmatic ASM cells were seeded onto RV-modulated and native ASM ECM. In the absence of RV infection, migration was greater in cells seeded onto asthmatic ECM than cells seeded onto ECM deposited by nonasthmatic cells. Furthermore, ECM deposited in response to RV infection of asthmatic and nonasthmatic ASM cells significantly enhanced the migration of ASM cells (Fig. 5).

The effect of RV-modulated ECM on ASM cell proliferation was assessed, as the ECM is also known to alter proliferation. RV-modulated nonasthmatic ASM ECM significantly decreased FBS-induced nonasthmatic ASM cell proliferation (4.5% and 4.9% at MOI 0.1 and 1, respectively) on day 3 (Fig. 6A).
Similarly, RV-modulated asthmatic ECM also decreased proliferation (3.9% at MOI 0.1 and 1) on day 3, and this decrease of nonasthmatic ASM cells persisted to day 5 (3.2% and 4.4% at MOI 0.1 and 1, respectively; Fig. 6B).

**DISCUSSION**

We report that in vitro RV infection of ASM cells from nonasthmatic and asthmatic subjects results in differential ECM deposition. RV infection increases the deposition of fibronectin and perlecan by nonasthmatic ASM cells, while fibronectin and collagen IV, but not perlecan, were increased in asthmatic cells. Increased ECM deposition was shown to be mediated by TLR3 and TLR7/8 agonists. Furthermore, RV modulation of ECM from nonasthmatic and asthmatic ASM cells increased cell migration but decreased cell proliferation. Thus the increase in ECM proteins and migration of ASM cells may play a role in the remodeling of the airway.

This is the first study that has investigated the direct effect of RV infection of ASM cell ECM deposition. Infection of nonasthmatic ASM cells by crude RV-16 showed increased deposition of fibronectin and perlecan. This response was shown to be virus-specific, as purified RV-16 and RV-2 also increased fibronectin and perlecan deposition and increased mRNA expression in nonasthmatic ASM cells. As RV-16 binds to the intercellular adhesion molecule-1 and RV-2 binds to the low-density lipoprotein receptor, this also demonstrates that increased ECM deposition was not specifically mediated via either receptor but, rather, through a common mechanism. TLRs are a group of pattern recognition receptors that are capable of detecting and responding to various components of pathogens. For example, TLR3 recognizes dsRNA, which occurs when ssRNA viruses, such as RV, replicate. Using the agonists poly I:C, imiquimod, and RV RNA, we were able to show that activation of TLR3 and TLR7/8 increased the deposition of ECM proteins, thus demonstrating that the cellular detection of viruses or the detection of viral replication activates ECM deposition. It is tempting to speculate that RV-induced ECM deposition is mediated via autocrine feedback of a profibrotic growth factor such as transforming growth factor-β (TGFβ). We believe that this is highly unlikely, as TGFβ is known to induce most, if not all, of the ECM components.

Fig. 3. Treatment of airway smooth muscle (ASM) cells with polyinosinic:polycytidylic acid (poly I:C), imiquimod, and RV-16 RNA increased fibronectin (A; n = 9) and perlecan (B; n = 9) deposition 48 h after Toll-like receptor agonist treatment. Values are means ± SE. ECM deposition was assessed by ECM ELISA. *P < 0.05, **P < 0.01, ***P < 0.005 vs. control.

Fig. 4. RV increased deposition of fibronectin (A; n = 9, *P < 0.05 at MOI 1) and collagen IV (B; n = 9, *P < 0.05 at MOI 1) 48 h after infection of asthmatic ASM cells. Values are means ± SE.

Fig. 5. RV-modulated ECM facilitates cell migration. RV-modulated nonasthmatic ASM ECM (n = 4) and asthmatic ASM ECM (n = 5) facilitated migration of virus-naïve nonasthmatic ASM cells compared with their respective control ECM. Values are means ± SE. Migration was assessed using Transwell inserts. #P < 0.05, nonasthmatic vs. asthmatic ECM. *P < 0.05, **P < 0.01 vs. respective control ECM.
that we measured in this study. For example, we previously found that collagen I is produced in response to TGFβ that we measured in this study. For example, we previously showed that RV modifies nonasthmatic ASM cells (18), and in the current study, we found no evidence of collagen I upregulation by RV. We do not know if RV activation of TLR3 or TLR7/8 results in ECM deposition in ASM cells. However, we recently showed that RV is detected via TLR3, and not TLR7/8, in primary human bronchial epithelial cells (37), but whether this is the case in ASM cells remains to be determined.

Reports suggest that fibronectin increases the migration (32) and proliferation of ASM cells, and given our observed increase in fibronectin by RV, we speculate that RV-modulated ECM would increase migration and proliferation of ASM cells. Nonasthmatic cells were used in these studies to eliminate the potential confounding effects of any phenotypic differences between cells from subjects with and without asthma. Our migration assays revealed facilitation of a higher level of migration in virus-naive ECM from asthmatic cells than nonasthmatic ASM cells. Furthermore, migration was increased on RV-modulated nonasthmatic and asthmatic ECM with respect to their uninfected ECM. Interestingly, there was a difference in migration between asthmatic and nonasthmatic ASM cells in virus-naive ECM, but not in RV-modulated ECM. The increase in migration in RV-modulated ECM may partially be a result of increased fibronectin (31); however, it is likely that other ECM components play a role in facilitating the changes. When we measured ASM cell proliferation, to our surprise, there was a small but significant decrease in proliferation on RV-modulated ECM from asthmatic and nonasthmatic ASM cells. These results are counterintuitive, given the increase in fibronectin that we found, and perhaps indicate that an ECM component that we did not measure is responsible for the changes.

It could be argued that RV-induced ECM changes observed in this study are modest and may be of limited significance. However, they are commensurate with findings of other studies. TGFβ stimulates ASM cells to produce twice the amount of fibronectin (6), and it produces a 70% increase in perlecan deposition in fibroblasts (9). Such limited increase in protein deposition was also reflected in gene expression, where collagen I was increased by 70% (6). In vivo studies comparing the amount of various ECM proteins between asthmatic and nonasthmatic airways showed a doubling of ECM components (12, 35); hence, our data are consistent with the in vivo changes.

Collagen IV exists as a trimer that forms between three of the six genetically different α-chains (α1–α6). The genes are arranged into pairs, α1–α2, α3–α4, and α5–α6, on three different chromosomes. These pairs of genes are highly unusual, in that they have a head-to-head orientation, and they share a bifunctional promoter region. The same stimulus can cause transcription of one of the gene pairs in some cell types, but not in others (cell-specific promoter plasticity) (40). In human ASM cells, the mechanisms that initiate transcription via the bifunctional promoter are not known; however, CCAAT enhancer-binding protein (C/EBP) and activator protein-1 binding sites have been identified in the promoter region. We previously showed that RV induces greater IL-6 in asthmatic ASM cells via differential recruitment of transcription factors to the promoter region, specifically transcription factors that bind to the C/EBP binding site. In asthmatic ASM cells, the transcription factor C/EBPα is absent (36). C/EBPα negatively controls gene transcription; therefore, when C/EBPα is absent, other protranscriptional members of the C/EBP family, such as C/EBPβ, can bind and initiate gene transcription. Therefore, it is plausible that the absence of C/EBPα in the asthmatic cells allows RV to induce the transcription and translation of collagen IV, while no upregulation occurs in the nonasthmatic cells. In contrast, the gene promoter region of perlecan is not as well characterized. It is known to contain a TGFβ-responsive element (13), but whether transcription factors such as nuclear factor 1 (NF-1), which can bind to this region, are also differentially regulated in asthmatic and nonasthmatic cells in response to RV is not known. Many studies have demonstrated intrinsic differences between asthmatic and nonasthmatic ASM cells (6, 18), and we observed differential ECM deposition by RV-infected nonasthmatic and asthmatic ASM cells. Furthermore, we previously demonstrated differential transcriptional regulation of interleukin-6 and -8 in RV-infected asthmatic and nonasthmatic ASM cells (28).

The strength of this study lies in the use of primary airway cells from a large number of subjects, which was because our cells are not immortal, and it is therefore not possible to repeat all the experiments in cells derived from the one subject. However, since the ASM cells in the nonasthmatic group are derived from resected lung tissue, explanted lung tissue, and bronchial biopsies of healthy subjects, this may be viewed as a
RHINOVIRUS-INDUCED ECM

limitation of our study. We are confident that the nonasthmatic group used in this study is appropriate, as in experiments in which we compared the response of ASM cells from the small number of normal subjects undergoing biopsy and, in the past, with donor lungs unsuitable for transplantation, we observed no differences in the response of these cells and the response of other nonasthmatic subjects compared with nonasthma cells from resected lung tissue (see Supplemental Fig. D2).

In summary, we have shown differential RV modulation of nonasthmatic and asthmatic ASM cell ECM. This feature of remodeling was shown to contribute to cell migration, potentially further enhancing airway remodeling. We hypothesize that RV infection of ASM cells in the subepithelial region modulates the ECM composition to facilitate the migration of ASM cells to the region of infection, thereby contributing to increased ASM bulk.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

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