An in vitro airway wall model of remodeling

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Choe, Melanie M., Peter H. S. Sporn, and Melody A. Swartz. An in vitro airway wall model of remodeling. *Am J Physiol Lung Cell Mol Physiol* 285: L427–L433, 2003; 10.1152/ajplung.00005.2003.—Recent studies have shown that mechanical forces on airway epithelial cells can induce upregulation of genes involved in airway remodeling in diseases such as asthma. However, the relevance of these responses to airway wall remodeling is still unclear since *1*) mechanotransduction is highly dependent on environment (e.g., matrix and other cell types) and *2*) inflammatory mediators, which strongly affect remodeling, are also present in asthma. To assess the effects of mechanical forces on the airway wall in a relevant three-dimensional inflammatory context, we have established a tissue culture model of the human airway wall that can be induced to undergo matrix remodeling. Our model contains differentiated human bronchial epithelial cells characterized by tight junctions, cilia formation, and mucus secretion atop a collagen gel embedded with human lung fibroblasts. We found that addition of activated eosinophils and the application of 50% strain to the same system increased the epithelial thickness compared with either condition alone, suggesting that mechanical strain affects airway wall remodeling synergistically with inflammation. This integrated model more closely mimics airway wall remodeling than single-cell, conditioned media, or even two-dimensional coculture systems and is relevant for examining the importance of mechanical strain on airway wall remodeling in an inflammatory environment, which may be crucial for understanding and treating pathologies such as asthma.

asthma; human bronchial epithelial cells; eosinophils; mechanical stress; airway inflammation

TISSUE REMODELING INVOLVES the alteration of compositional, architectural, and organizational components that ultimately affect tissue function. Although remodeling occurs during development and healing to reinstate proper tissue function, it can often be the result of injury or the hallmark of disease. In asthma, remodeling of the airway contributes to airflow obstruction. The most prominent remodeling feature is the thickening of airway wall, which is reported to increase 10–100% in nonfatal and 50–300% in fatal asthma (14, 18). Specific features of airway remodeling in asthma include subepithelial fibrosis, hypertrophy, and/or hyperplasia of smooth muscle, mucus metaplasia, infiltration of inflammatory cells, myofibroblast hyperplasia, edema, and angiogenesis (7, 9, 16). Although the morphological changes in remodeled airways have been well characterized, the mechanisms behind the cause and progression are not well understood as multiple factors such as inflammation, mechanical stress, and cell-cell and cell-extracellular matrix (ECM) interactions all interact in complex ways and may act synergistically to contribute to remodeling.

Recent studies have shown that compressive forces on epithelial cells can induce factors involved in remodeling. In an asthmatic airway, hyperresponsive smooth muscle cells shorten to a greater extent (21), causing the mucosal and submucosal layers to buckle into the lumen, resulting in a complex mechanical environment that may include compression, bending, and shear. Also, compressive stress on epithelial cells, in the physiological range produced by smooth muscle cells of >10–40 cmH_2O (11, 34), has been shown to upregulate transformed growth factor-β, endothelin-1, and early growth response-1 (29) and elicit unstressed, cocultured fibroblasts to upregulate collagen type III, fibronectin, and matrix metalloproteinase-9/tissue inhibitor of metalloproteinase-1 (MMP-9/TIMP-1) ratio (32), all factors associated with remodeling. Furthermore, compressive stress of 20 cmH_2O in the same system resulted in a 10% strain of epithelial cell height, indicating that deformation may be an important mechanosensory mechanism (6). These results, among others, suggest that mechanical strain on airway epithelial cells can produce factors that lead to airway wall remodeling.

There are at least three issues that limit the relevance of such results on understanding remodeling diseases such as asthma. First, inflammatory cells and mediators are present in the airway in asthma that may affect remodeling. Second, the airway wall is composed of cells organized in a three-dimensional matrix, which is important in cellular responses, especially in relaying mechanical signals that mainly occur via ECM-integrin interactions (1, 30). Third, forces generated by smooth muscle cells can affect not only epithelial cells but also fibroblasts that reside in the connec-

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tive tissue below the epithelium (10, 23), and cell-cell communication may play an important role in directing strain-induced remodeling (32). To investigate the interplay among some of the key factors that contribute to long-term remodeling such as mechanical strain and inflammation, we developed a three-dimensional in vitro airway wall model representing the airway epithelium and underlying connective tissue. Our model contains the following features: *1*) fibroblasts and epithelial cells cocultured in an ECM, *2*) physiologically relevant phenotypes exhibited by the two cell types, and *3*) the ability to manipulate the specific biochemical and biomechanical environments. This model has some advantages over both in vivo and traditional cell culture models: variables such as strain and inflammation are inseparable in an in vivo model, whereas monolayer or single-cell culture models lack the organization and complexity of a cellular community. Furthermore, our model is unique in that mechanical strain can be applied to a three-dimensional coculture system; this is particularly relevant in remodeling studies since three-dimensional cell-ECM contacts (e.g., integrins) likely play key roles in mechanotransduction (1, 30).

The model consists of fibroblasts suspended in a collagen matrix with differentiated epithelial cells on the surface in a device that prevents lateral contraction of the gel during culture. We determined the effects of mechanical strain, activated eosinophils, and a combination of the two, on visible evidence of matrix remodeling (via histology and immunohistochemistry). The mechanical strain was imposed by a lateral strain of 50%. Inflammatory mediators were introduced by placing freshly isolated and activated human eosinophils on the epithelial surface, since these cells are commonly present in significant numbers in asthmatic airways (31) and are known to release preformed granules and lipid mediators leading to symptoms of inflammation (2, 3, 25, 33). In characterization, the model exhibits a differentiated epithelium and structural organization similar to that seen in vivo. Additionally, it is a functional model of remodeling, where additional measures of remodeling such as collagen synthesis (e.g., [^{3}H]proline incorporation), matrix degradation (e.g., in situ zymography, [^{3}H]proline pulse-chase), and specific protein production (e.g., Western blots for a range of matrix proteins, MMPs, and TIMPs) can be readily employed to characterize remodeling. In an example of its use, we found that epithelial thickening was induced by a combination of 50% mechanical strain and activated eosinophils but not by either one alone. These findings lend new insight to the synergistic role of mechanical strain and inflammation in remodeling as well as demonstrate the versatile features of the model in elucidating the mechanisms of chronic remodeling in diseases such as asthma.

MATERIALS AND METHODS

Cell culture. IMR-90 human fetal lung fibroblasts (HLF) from American Type Culture Collection (Manassas, VA) were cultured in α-MEM supplemented with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY) and 1% penicillin/streptomycin (Sigma, St. Louis, MO). HLFs at *passages 13–15* were used in the model.

Normal human bronchial epithelial cells (HBEC) (Clonetics, Walkersville, MD) were expanded in bronchial epithelial growth medium (BEGM, Clonetics) and used at *passage 3* in the model.

Model design. A schematic of the model is shown in Fig. 1. To facilitate the application of a composite mechanical stress such as that seen in a buckled airway, the culturing vessel includes a built-in strain applicator made of one-quarter inch-thick porous polyethylene (PE) sheets (Small Parts, Miami Lakes, FL) as depicted in Fig. 1*A*. To support the gel in the PE wells with an air-liquid interface (ALI), a porous polycarbonate filter with 5-μm pores (Osmonics, Kent, WA) was attached to the bottom of the well with silicone glue. For unstrained controls, single PE rectangular rings of the same dimensions were constructed.

The PE wells were placed in wells designed for standard microscope slides (Nalge Nunc International, Naperville, IL) and filled with a suspension of HLFs at 5×10^5 cells/ml in 2.5 mg/ml of collagen [isolated from rat tail tendons as previously described (26)]. The surface was then coated with an additional thin (750 μm) layer of 2.5 mg/ml of collagen. This HLF suspension in collagen was introduced to media consisting of one part DMEM (GIBCO-BRL, Grand Island, NY) and one part BEGM to just cover the surface of the gel, and HBECs were seeded on the surface at 2.5×10^5 cells/cm^2.
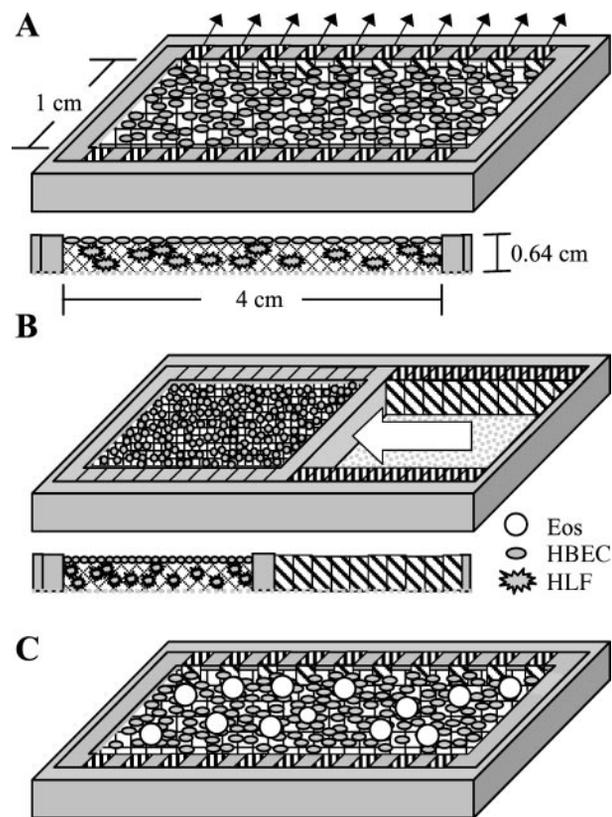

Fig. 1. Schematic of the airway wall model. *A*: the system consists of porous polyethylene frames filled with fibroblast (HLF)-suspended collagen gel. Epithelial cells (HBEC) are seeded on top. The environment of the model can be altered to introduce strain (*B*) by removing alternating (hatched) pegs and reducing the total area and/or inflammatory mediators (*C*) such as eosinophils (Eos). Side views are illustrated in *A* and *B*.

After 2 h, additional media were added to culture in submersion for 1 wk to allow HBECs to reach confluence. To achieve ALI, the cultures were set atop strips of 1.6-mm-thick PE at each end of the device, and media volume was decreased to expose the HBEC surface to air. ALI was maintained to allow differentiation of HBECs, and the surface was rinsed with PBS daily. After 7 days of culture in ALI, strain, eosinophils, both, or neither was imposed on the system. Strain was imposed by removing 10 spacers intermittently from the inner ring and placing them between the inner and outer rings (Fig. 1*B*). This led to 50% strain, which was maintained for a period of 48 h. The magnitude of strain was chosen to mimic smooth muscle shortening, which can range anywhere between 30 and 70% in nonasthmatic and asthmatic airways (15, 21). The duration was chosen to allow visible changes in matrix architecture (via histology) to occur, which was determined in pilot studies.

Model characterization. The model was characterized for epithelial cell differentiation and tight junction formation, mucus secretion, and cell organization. The growth of cilia by columnar epithelial cells was observed with a scanning electron microscope (SEM; Hitachi 4500N) using standard sample preparation. Briefly, the model was fixed in 2.5% glutaraldehyde for 2 h, dehydrated in a series of ethanol, and critical point dried with CO_2 before coating with gold:palladium (60:40). Micrographs were taken of the epithelial surface at $\times 1,000$ and $\times 10,000$ magnifications.

To quantify mucus production, epithelial secretions were evaluated over a 10-day period by blotting surface rinses and staining the blots with alcian blue, which stains type I mucin. First, the surface of the epithelial layer (after *days 1–10* of ALI) was rinsed with 0.5 ml of PBS. These rinses were transferred into a vacuum-driven Dot Blot apparatus (Bio-Rad, Hercules, CA) where they were concentrated into dots onto nitrocellulose membranes. The membranes were then stained in 1% alcian blue 8 GX (Sigma) in 3% acetic acid solution, pH 2.5, according to Pon et al. (28). The blots were scanned (ScanJet 4200C, Hewlett Packard), and the color intensity of each dot was measured using Scion Image (NIH, Bethesda, MD).

The presence of tight junctions was confirmed by immunostaining for occludin, a transmembrane protein localized to tight junctions (24). To achieve this, the surface epithelial layer was removed and immunostained; this was facilitated by placing a polycarbonate filter membrane with a pore size of 0.8 μm (Osmonics), coated with the same rat tail tendon collagen as before, atop the HLF-seeded collagen before seeding the HBECs. Thus the filter served as a physical support that could be used later to separate the epithelial layer from the fibroblast-populated collagen gel. After 7 days of ALI, a scalpel was used to cut around the circumference of the membrane and carefully lift it from the fibroblast culture. This epithelial layer was transferred to a glass slide, fixed in 1:1 acetone:methanol, blocked with 1% BSA (Sigma), and incubated with a FITC-conjugated mouse anti-human occludin antibody (Zymed Laboratories, San Francisco, CA) at a concentration of 1 μg/ml. The nuclei were counterstained with 4′,6-diamidine-2-phenylindole dihydrochloride (DAPI) containing mounting medium (Vector Laboratories, Burlingame, CA).

Several other characteristics of the model were assessed, including cellular morphology, organization, and matrix protein production. Fibroblast organization and morphology were examined in histological cross sections as well as with SEM. To examine cellular organization, paraffin-embedded, formalin-fixed samples were thin sectioned and stained with hematoxylin and eosin (Sigma) after 10 days in ALI. For

fibroblast morphology, cells within the collagen were exposed by tearing the tissue after critical point drying. SEM as detailed above was then used to view the cells in the matrix. Finally, matrix components such as fibronectin, initially absent from the system, were examined via immunofluorescence. Frozen sections were fixed in acetone and methanol and incubated overnight at 4°C in 0.25 μg/ml of mouse anti-human fibronectin antibody (Transduction Laboratories, Lexington, KY) after blocking in 10% solution of rabbit serum (Jackson ImmunoResearch Laboratories, West Grove, PA). The sections were then incubated in 2 μg/ml of biotinylated rabbit anti-mouse secondary antibody (Dako, Glostrup, Denmark) and mounted with DAPI containing mounting medium (Vector Laboratories).

To assess collagen within the model after the introduction of strain and/or inflammation, 10-μm paraffin-embedded sections were stained with van Gieson's stain and counterstained with Weigart's hematoxylin.

Inflammatory environment. To introduce inflammatory mediators onto the model, freshly isolated and activated human eosinophils were placed on the apical surface (Fig. 1*C*). Eosinophils were obtained from venous blood of human volunteers, as previously described (4). The protocol for use of human subjects was approved by the Institutional Review Board of Northwestern University. Blood donors were nonsmokers and were not taking oral or inhaled steroids, aspirin, nonsteroidal anti-inflammatory drugs, leukotriene synthesis inhibitors, or leukotriene receptor antagonists. Briefly, heparinized venous blood was centrifuged through Percoll (Amersham Pharmacia Biotech, Piscataway, NJ), density 1.084 g/ml, and erythrocytes were removed by hypotonic lysis. The remaining granulocytic fraction was washed, counted, and incubated on ice for 30 min with MACS CD16 MicroBeads (Miltenyi Biotec, Auburn, CA). The granulocyte suspension was then applied to type CS magnetic columns (Miltenyi Biotec) in a 0.6 Tesla magnetic field, and the eluate containing eosinophils was collected. Eluates were washed and resuspended in RPMI 1640 containing 10% fetal calf serum. The resulting cell suspensions were 97 ± 1% pure eosinophils and were ≥98% viable by trypan blue staining.

After 7 days of epithelial ALI, eosinophils were seeded atop the epithelium at a ratio of 1:10 (eosinophils:HBECs). This ratio was determined from pilot experiments that showed that higher ratios of eosinophils to HBECs decreased epithelial cell viability over a period of 4 days. Eosinophils were introduced to the epithelial surface after 7 days in ALI and allowed to adhere to the epithelium in submersion for 2 h. The model was then returned to ALI and stimulated with calcium ionophore (A-23187) at 2 μM (Calbiochem, San Diego, CA).

Experimental conditions. Four experimental conditions were considered in the evaluation of remodeling, cocultures with: *1*) no stimulation, *2*) 50% strain, *3*) activated eosinophils, and *4*) both activated eosinophils and 50% strain, designated hereafter as control (*group C*), strain (*group S*), eosinophil (*group E*), and strain plus eosinophil (*group S + E*), respectively. Three experiments were performed for each experimental condition.

Statistical analysis. Results are expressed as means ± SE. Mean values were compared using Tukey's method and considered significant for $P < 0.05$.

RESULTS

Model characterization. After 1 day of ALI culture (following 7 days of submersion culture), the apical surface of the model showed a confluent epithelial cell
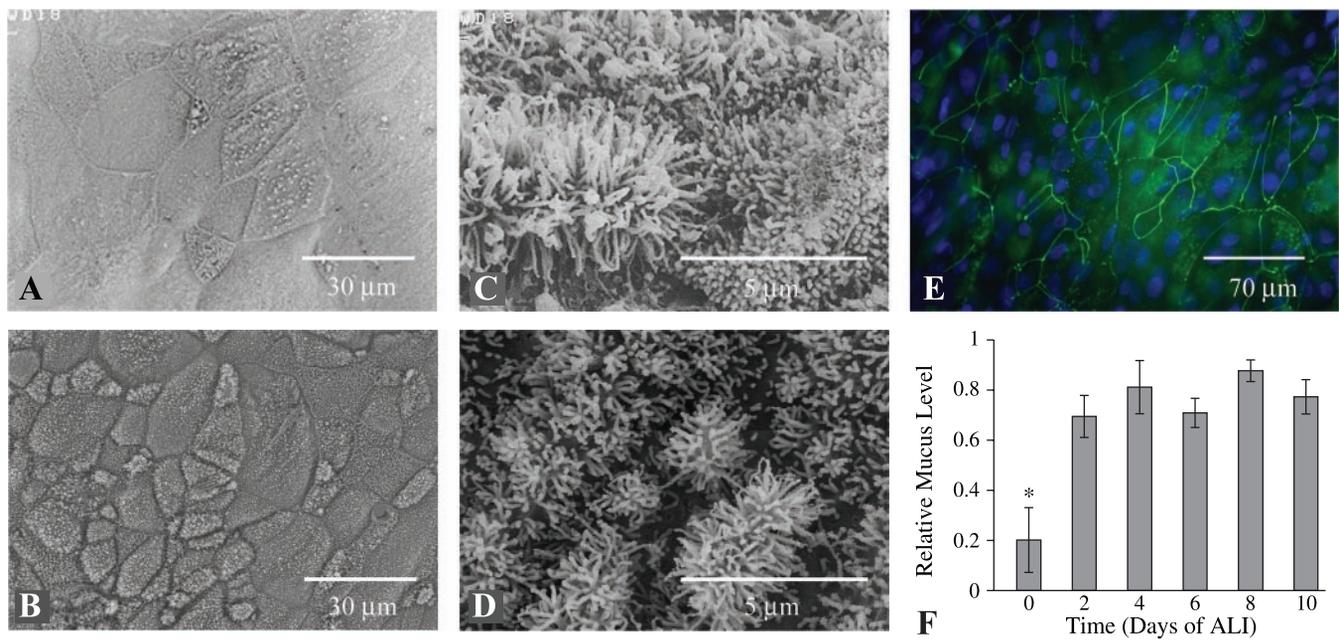
Fig. 2. Epithelial characterization. The apical surface of the model was characterized by tightly packed epithelial cells at *day 1* (*A*) and *day 10* (*B*) of air-liquid interface (ALI). Under higher magnification, cilia growth was seen in *C* and *D* on the respective days. *E*: immunofluorescent staining shows the presence of occludin (green, nuclei blue). *F*: consistent mucus production was observed over 10 days of ALI (*$P < 0.05$).

layer with a thick coat of cilia (Fig. 2, *A* and *C*); these attributes were maintained over 10 days (Fig. 2, *B* and *D*). Evidence of tight junctions was observed by immunofluorescent staining of occludin (Fig. 2*E*). In addition, by day 2 of ALI culture, consistent mucus production was observed (Fig. 2*F*).

Hematoxylin and eosin staining revealed epithelial cells packed on the surface and fibroblasts stretched and anchored throughout the collagen matrix (Fig. 3*A*).
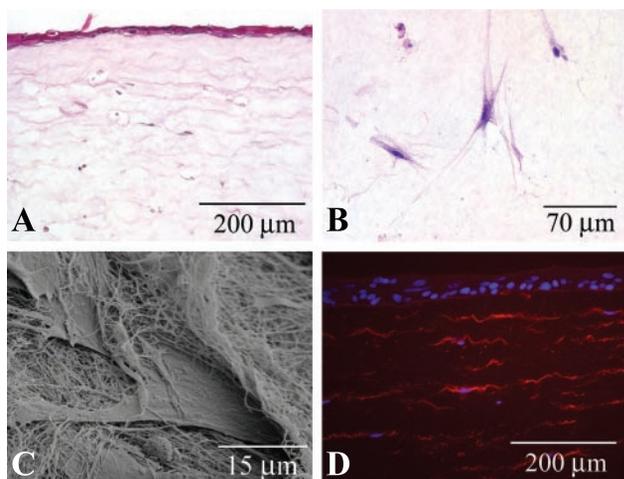

Fig. 3. Cellular organization within the airway wall model after 10 days of ALI. *A*: hematoxylin and eosin (H&E) staining reveals human bronchial epithelial cells confined to the apical surface of the model. *B*: H&E staining shows fibroblasts stretched within the subepithelial matrix. *C*: scanning electron microscope shows a fibroblast anchored to extracellular matrix fibrils. *D*: immunofluorescent staining of a 10-μm section shows fibronectin (red) near fibroblasts (all nuclei are stained blue with 4′,6-diamidine-2-phenylindole dihydrochloride).

Under higher magnification, fibroblasts were seen spread within the gel with projections extending out from the cell body (Fig. 3*B*). This was also observed by SEM in Fig. 3*C*, which shows an individual fibroblast embedded within a matrix of collagen fibrils. In a matrix originally comprising mainly type I collagen, fibronectin (a fibroblast chemotactic factor) (13, 17) was found localized near fibroblasts (Fig. 3*D*). Other ECM proteins, including type IV collagen, were also identified in the matrix by immunofluorescence microscopy (data not shown).

In *groups S* and *S + E*, a static strain of 50% was applied in the lateral direction of the model (i.e., reducing the surface area of the system by 50%) to mimic the buckling pattern present in a constricted airway. This lateral compression was maintained for 48 h, introducing complex (e.g., bending, compressive, and shear) straining patterns to the system (Fig. 4) compared with a system without strain (Fig. 3*A*).

Effects of mechanical strain and inflammation. The effects of strain and inflammatory cells on the models were assessed after 48 h by direct examination of histological thin sections. Sections were stained using van Gieson's solution to compare the architecture of the collagen matrix. Several interesting structural changes were observed consistently among the three sets of identical experiments we performed. *Group S + E* (Fig. 5*D*) displayed an increase in the thickness of the epithelial cell layer compared with other conditions. Additionally, eosinophils with bilobed nuclei can be seen on the apical surface of the epithelium in Fig. 5*D*. These features were not observed in *groups S* (Fig. 5*B*) or *E* (Fig. 5*C*). *Group S + E* (Fig. 5*D*) also showed different buckling patterns than *group S* (Figs. 4 and
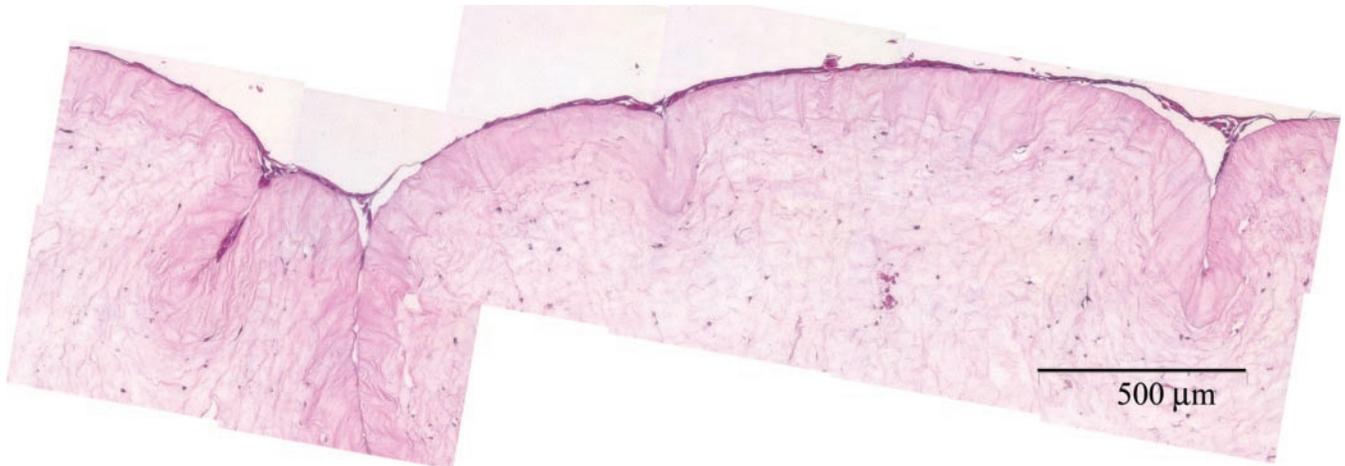
Fig. 4. Cross section of strained airway wall model (*group S*). A thin section, fixed under 50% strain and stained with H&E, shows the complex buckling pattern similar to that seen in the airway wall.

5*B*): instead of the close epithelium-to-epithelium folds, shallow dips are observed on the surface.

DISCUSSION

We have developed a model of the airway wall that has physiologically relevant cellular structures and ECM components and that can allow investigations of remodeling due to mechanical strain and/or inflammatory mediators. Some of the functional phenotypes of the epithelial layer include tightly packed cells that form a barrier to external elements with tight intercellular junctions, cilia growth, and mucus secretion. In
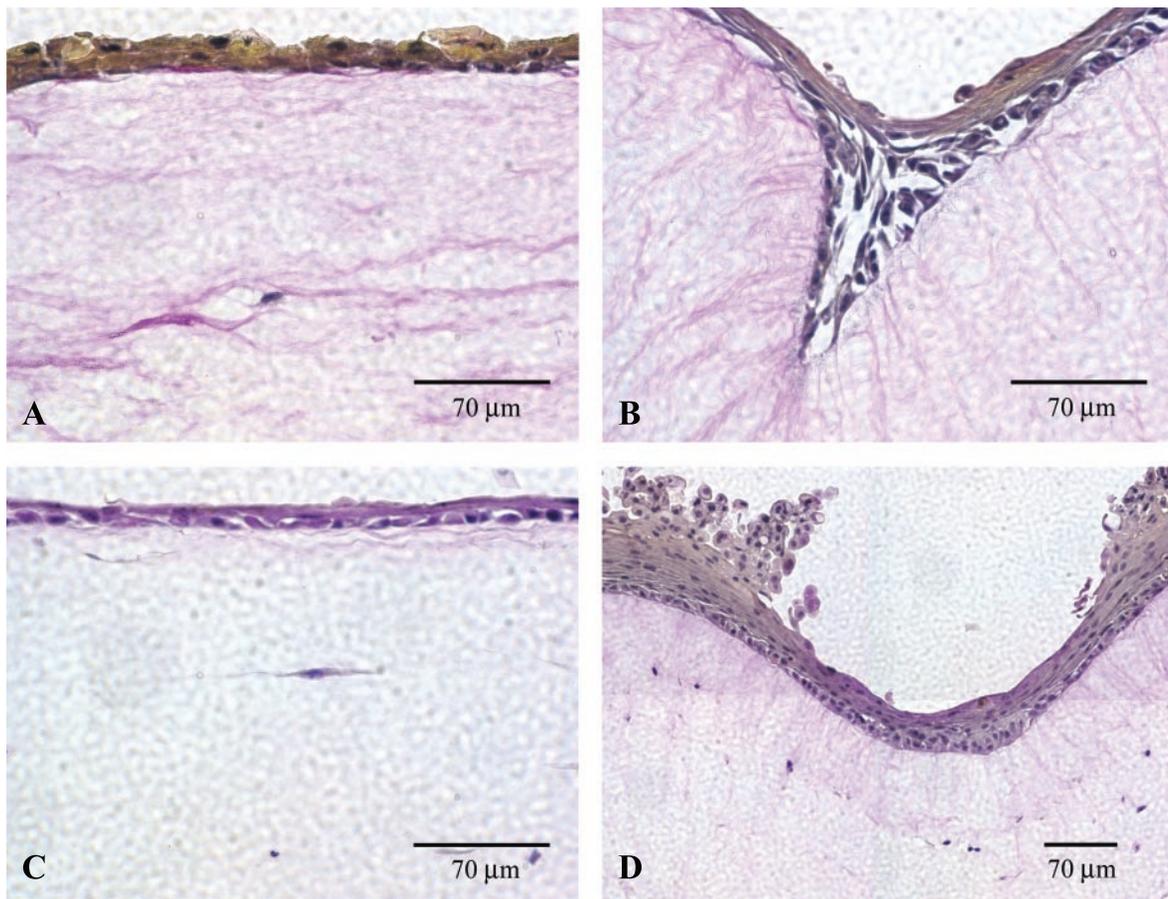

Fig. 5. Effect of strain and inflammation on structure. Van Gieson and hematoxylin stains were used to stain collagen pink and nuclei blue/black. *A*: static control (*group C*). *B*: strained model (*group S*) with a buckled surface. *C*: static model with eosinophils (*group E*) with decreased collagen density. *D*: strain and eosinophil model (*group S + E*) that resulted in epithelial hyperplasia.

addition, these cells communicate with other cell types, such as fibroblasts and inflammatory cells, via biochemical signals and the ECM they cohabit to alter their environment accordingly. These features are all essential to the proper functioning of the bronchial wall.

The subepithelial layer contains fibroblast-rich connective tissue that maintains the integrity of the ECM. Importantly, the epithelial cells and fibroblast communicate the state of their environment via their shared matrix; thus remodeling responses to biochemical and/or mechanical perturbations to the airway wall would be complex and interdependent on cell-cell and cell-ECM interactions. The mechanisms behind remodeling are not well understood, but inflammation is thought to be a dominant factor while mechanical stress may also contribute, although it is not clear to what extent (8, 16, 22, 32). In our model, the mechanical and inflammatory environments can be independently manipulated, allowing us to examine their individual and combined effects on the complex remodeling response of the model.

To mimic the mechanical environment found in a constricted airway, lateral compressive strain was imposed on the model, which induced buckling folds similar to that seen in vivo (34). This compression resulted in matrix deformation that affected both the HBECs and HLFs, which were in direct communication via the ECM. This is critical to examining the system response to mechanical strain (where the system includes the two cell types and the ECM) since the complex interactions between cells and their ECM, as well as between different cell types that share the same ECM, can strongly affect cellular response to mechanical strain, inflammatory mediators, or any other stimulant. It is also known that cellular responses are dependent on matrix composition and dimension. For example, Liu et al. (20) observed that fibroblast proliferation is decreased in two-dimensional vs. three-dimensional stretched models.

In addition to mechanical strain imposed by bronchoconstriction, inflammatory cells infiltrate the airway wall in asthma. Eosinophils are the predominant inflammatory cells found in asthmatic airways (31). Thus to mimic this inflammatory state, activated eosinophils were added to our model. Stimulated eosinophils release inflammatory mediators, such as leukotriene C_4, which is known to promote epithelial cell proliferation (19) and collagen synthesis by fibroblasts (27). Our system responded to both inflammatory and mechanical changes in its environment, representing some functional aspects of an airway wall. Eosinophil and strain had a synergistic effect in that epithelial hyperplasia was observed only in *group S + E*. The folding pattern, therefore, may not have been as pronounced in *group S + E* compared with that of *group S*, strain alone. This was consistent with a recent study by Wiggs et al. (34), who found that a thickened airway wall, inclusive of the epithelium, leads to larger and fewer folds in the airway and greater obstruction than a normal thickness airway wall.

Although the human airway includes smooth muscle cells, mucus glands, blood vessels, and various layers of matrix as well as epithelial cells and fibroblasts, we included only epithelial cells and fibroblasts in our model. In doing so, we assume that the dominant effect of smooth muscle cells is to impose the mechanical stress (which was mimicked in our model by an external strain device) rather than to mediate subepithelial remodeling directly by molecular mediators. This is obviously a limiting assumption and thus we are not able to include the known contributions of smooth muscle cells in affecting the airway wall "society" in response to stimulants such as mechanical stress or inflammatory cells (5, 12). However, these cell types could be included in a future modification of the model.

In future investigations, modifications will be made to study the importance of oscillatory strain, since the lung is an organ under constant dynamic stress. Additionally, other inflammatory cells and specific mediators can be introduced and their mechanistic role in remodeling identified. The methods used in this study for the investigation of remodeling were qualitative. Further analysis of specific matrix components known to be upregulated in asthmatic airways, such as collagen types III and V and fibronectin, including spatial and temporal data, can be investigated.

In summary, our new model addresses the need for a more comprehensive in vitro model of the airway wall as a dynamic system. Because it is able to support multiple cell types without contraction of the matrix to observe and elucidate the long-term remodeling effects of factors such as inflammation and mechanical stress, this model is relevant for studying remodeling events associated with asthma.

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