Effects of dynamic compression on lentiviral transduction in an in vitro airway wall model

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Tomei AA, Choe MM, Swartz MA. Effects of dynamic compression on lentiviral transduction in an in vitro airway wall model. Am J Physiol Lung Cell Mol Physiol 294: L79–L86, 2008. First published November 16, 2007; doi:10.1152/ajplung.00062.2007.—Asthmatic patients are more susceptible to viral infection, and we asked whether dynamic strain on the airway wall (such as that associated with bronchoconstriction) would influence the rate of viral infection of the epithelial and subepithelial cells. To address this, we characterized the barrier function of a three-dimensional culture model of the bronchial airway wall mucosa, modified the culture conditions for optimization of ciliogenesis, and compared epithelial and subepithelial green fluorescent protein (GFP) transduction by a pWpts-GFP lentivirus, pseudotyped with VSV-G, under static vs. dynamic conditions. The model consisted of human lung fibroblasts, bronchial epithelial cells, and a type I collagen matrix, and after 21 days of culture at air liquid interface, it exhibited a pseudostratified epithelium comprised of basal cells, mucus-secreting cells, and ciliated columnar cells with beating cilia. Microparticle tracking revealed partial coordination of mucociliary transport among groups of cells. Slow dynamic compression of the airway wall model (15% strain at 0.1 Hz over 3 days) substantially enhanced GFP transduction of epithelial cells and underlying fibroblasts. Fibroblast-only controls showed a similar degree of transduction enhancement when undergoing dynamic strain, suggesting enhanced transport through the matrix. Tight junction loss in the epithelium after mechanical stress was observed by immunostaining. We conclude that dynamic compressive strain such as that associated with bronchoconstriction may promote transepithelial transport and enhance viral transgene delivery to epithelial and subepithelial cells. This finding has significance for asthma pathophysiology as well as for designing delivery strategies of viral gene therapies to the airways.

Address for reprint requests and other correspondence: M. A. Swartz, Institute of Bioengineering, SV-LMBM, Station 15, Ecole Polytechnique Fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland (e-mail: melody.swartz@epfl.ch).

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constant perimeter (40, 41, 58, 74). In asthma, bronchoconstriction occurs more frequently, due to hyperresponsive SMCs (24, 25), and at greater magnitude, due to both hyperreactive SMCs and the remodeled airway wall (41, 57, 58, 74), which lead to fewer and deeper folds in the buckled epithelial layer in response to constriction.

Although in vitro airway wall models to mimic various features of the bronchial airways have been developed by ourselves and others (10–13, 20, 44, 50, 51, 64, 71), their degree of ciliogenesis, a critical barrier function, is often low. Using a 3D fibroblast-epithelial coculture model of the human airway wall mucosa that can undergo 3D lateral dynamic strain as would occur during bronchoconstriction (11), we first optimized culture conditions for key functional features, including a pseudostratified epithelium, mucus secretion, and an extensive carpet of beating cilia that display some degree of cell-to-cell synchrony. We then used this model to demonstrate that dynamic compressive strain substantially enhances viral transport and infection using a green fluorescent protein (GFP)-containing lentiviral vector. Our results demonstrate that mechanical stress associated with bronchoconstriction may promote viral transport to epithelial and subepithelial cells and thus exacerbate the susceptibility of the airway wall to viral infection.

MATERIALS AND METHODS

Cell culture. Human fetal lung fibroblasts (HLFs; IMR-90, ATCC, Manassas, VA) were expanded in αMEM (GIBCO) supplemented with 10% FBS (Invitrogen, Paisley, UK) and 1% penicillin-streptomycin-amphotericin (Invitrogen) and used at passage 16. Normal human bronchial epithelial cells (NHBE; Cambrex, Verviers, Belgium) were expanded in supplemented bronchial epithelial growth medium (Cambrex) supplemented with 1.5 μg/ml BSA (Invitrogen) and 15 ng/ml retinoic acid (Sigma) and used at passage 3.

Coculture medium composition was varied to optimize ciliogenesis based on previously published protocols (12, 45, 55). The final optimized coculture medium, used for all the data presented in this study (medium A) consisted of 50% DMEM (low glucose, Invitrogen), 50% bronchial epithelial basal medium, 200 μg/ml bovine pituitary extract (Pel-Freeze Biologicals, Rogers, AR), 0.5 ng/ml human recombinant epidermal growth factor (Collaborative Research, Lexington, MA), 5 μg/ml hydrocortisone, 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 30 ng/ml retinoic acid, 6.5 ng/ml triiodothyronine, 1.5 μg/ml BSA, 12 μg/ml calcium chloride, 30 ng/ml ethanolamine, 0.3 μg/ml phosphoethanolamine, 50 μg/ml gentamicin, and 50 ng/ml amphotericin-B (all from Sigma). For optimization, we varied the concentration of retinoic acid, bovine pituitary extract, calcium, ethanolamine, phosphoethanolamine, and human recombinant epidermal growth factor and looked at ciliogenesis after 7, 14, and 21 days using scanning electron microscopy (see Epithelial characterization). For data presentation, we compared two of the different medium compositions tested and later refer to these as medium A (the optimal medium) and medium B (that used in our previous publications; Refs. 11, 12).

Airway wall model. The dynamic strain culture device (Fig. 1B) has been previously described (11). HLFs were suspended at 500,000 cells/ml in 2.5 mg/ml type I collagen (BD Biosciences, Basel, Switzerland) and allowed to polymerize at 37°C, 5% CO2 within the culture device. The gel surface was then coated with a thin layer (~50 μl/cm2) of 2.5 mg/ml acellular collagen to serve as a scaffold for epithelial cell remodeling and secretion of the cells’ own basement membrane, and after polymerization, the gel surface was covered with coculture medium. NHBEs were then seeded atop the collagen-HLF culture at 2,500 cells/mm2 and allowed to attach for 2 h. Finally, the model was submerged in coculture medium for 7–9 days with medium changes every 2 days. The air liquid interface (ALI) was established after epithelial confluence was reached, and the model was maintained for up to 3 wk to allow epithelial differentiation into a pseudostatified layer. During medium changes, the epithelium was washed with 500 μl of HBSS (Invitrogen) and the lavage was stored at −80°C for later detection of types I and III mucin.

Epithelial characterization. The model was characterized for relevant barrier function properties, including epithelial morphology, degree of differentiation and stratification, ciliogenesis and mucociliary transport, and mucus secretion. Samples were fixed and cryosectioned into 4% paraformaldehyde. Histological staining on 6-μm sections was used to determine overall morphology and to identify mucin-producing cells by Alcian blue (Sigma) and was imaged under an Olympus CX41 microscope with the Olympus DP12-2 camera. To label basal...
epithelial cells, rabbit anti-human keratin 14 antibody (1:250, Covance, Berkeley, CA) and AlexaFluor 594-conjugated secondary IgG (1:500, Molecular Probes, Eugene, OR) were used on 10-µm-thick sections. AlexaFluor 488-conjugated phallolidin (1:50, Molecular Probes) was used to examine the actin cytoskeletal structure of columnar cells. Images were taken using a Zeiss Axiovert 200M fluorescence microscope and AxioCam MRm camera. Finally, tight junctions were visualized using a mouse anti-human occludin antibody (1 µg/ml, Zymed Laboratories, South San Francisco, CA) on 70-µm-thick sections and were imaged under a confocal microscope.

Scanning electron microscopy was used to characterize ciliogenesis after various times in ALI culture. Samples were fixed in 2% gluteraldehyde (Sigma) for 2 h, washed in Sorensen buffer solution, and treated with 1% osmium tetroxide for 1 h. Then, they were dehydrated in a sequence from 25 to 100% ethanol, dried by CO2 critical point, coated with 20 nm of gold, and imaged with the Jeol JSM-6300F microscope equipped with the Mamiya 6×7 Joel UHR (TMX 120 ISO100) camera.

Protein dot blot was used to confirm mucin production as described previously (12). Briefly, samples and standards (mucin types I and III, Sigma) were filtered through a nitrocellulose membrane (NitroPure, GE Osmonics, Minnetonka, MN) using Bio-Dot SF apparatus (Bio-Rad, Hercules, CA). The membrane was stained with Alcian blue (for type III mucin) or periodic acid-Schiff (for type I mucin) and analyzed with the ChemiDoc XRS system (Bio-Rad).

To examine mucociliary transport function, fluorescent microspheres were tracked using long-exposure fluorescence microscopy. Without the surface being washed, 20 µl of 1-µm-diameter yellow-green polystyrene microspheres (4 × 107 particles/ml, FluoroSphere, Molecular Probes) were gently pipetted onto the epithelial surface. After being allowed to settle for 2 h at 37°C and 5% CO2 in a humidified environmental chamber on the microscope stage, the samples were observed. Images were taken at a 16-s exposure time and evaluated using ImageJ software (National Institutes of Health, Bethesda, MD), and the average velocity was calculated for each particle (total trajectory length/exposure time). Only particles that moved were chosen for calculations. In addition, AVI-format movies were created to verify the microsphere movements.

Lentiviral vectors were produced via a transient expression system consisting of three genetic elements: a transfer construct (pWpts-GFP), a packaging construct (pCMVR8.74), and an envelope expression construct (pMD2.G); all were kind gifts of D. Trono. These expression constructs were maintained in the form of bacterial plasmids and were transfected into HEK293T cells to produce replication-defective lentivirus stocks. A volume of virus solution having a multiplicity of infection equal to 10 for the NHBE was used to infect NHBE blasts were infected (Fig. 4A). Furthermore, we repeated the studies in epithelial-free (fibroblast-only) cultures and found a similar enhancement of infection with dynamic strain (Fig. 4, B–D), again indicating increased transport with strain.

Finally, to determine whether the barrier function of the epithelium was compromised by the dynamic strain, as has been reported to occur in vivo (35), we stained 70-µm-thick sections for occludin, a primary tight junctional protein in the epithelium. Because of the 3D nature of the model, we imaged these thick sections using confocal microscopy and saw that while epithelial cell-cell interfaces could be clearly delineated in static controls, dynamically compressed samples exhibited more diffuse and weaker staining, and cell-cell interfaces could not be delineated (Fig. 5A). Quantification of images revealed that the amount of occludin staining (% of occludin-positive area; Fig. 5B) was decreased in strained samples.
DISCUSSION

The incidence of asthma is continually increasing, and together with its secondary effects, which include increased susceptibility to viral infection (2, 9, 16, 22, 26, 36, 61), constitutes an enormous public health problem. Inflammatory cells and cytokines, bronchoconstriction, and airway wall remodeling all act synergistically to exacerbate the disease, and the enhanced epithelial susceptibility to viral pathogens and particulate matters also aggravates symptoms. It is likely that this increased susceptibility is at least partly caused by epithelial activation and damage and by factors including cytokines secreted by mesenchymal and inflammatory cells, which in turn cause loss of barrier function. However, asthmatic airways also experience compressive strain due to frequent bronchocon-

Fig. 2. The epithelium is well differentiated after 21 days in air liquid interface (ALI). A and B: representative images of hematoxilin/alcian blue (A) and fast red nuclear stain/alcian blue (B) showing differentiated goblet cells (bright blue) among the columnar epithelial cells of the pseudostratified epithelium. There was significant variability of the epithelial thickness within each sample. Scale bar = 200 µm. C: basal cells (keratin-14, red) and the F-actin cytoskeleton of columnar cells (phalloidin, green) seen in a 10-µm section of the epithelial surface. Blue, nuclei (DAPI); scale bar = 20 µm. D: protein dot blot for mucin type I (light grey) and type III (dark grey) from epithelial washings. Values correspond to the amount secreted by 1 mm² of epithelium.

Fig. 3. Ciliogenesis and mucociliary function. A and B: scanning electron micrographs illustrate the extent of ciliogenesis at 7, 14, and 21 days of culture at ALI in the optimal medium composition medium A. Scale bars: A = 10 µm; B = 1 µm. C and D: micrographs of epithelial surface from less optimal medium B, used in previous studies (11, 12). Scale bars: C = 10 µm; D = 1 µm. All images in A–D are representative of cilia coverage within each sample. E: mucociliary transport as revealed by 16-s trajectories of 1-µm microspheres on the epithelium; 4 different experiments are shown to illustrate the range of variability seen. Regions of coordinated or synchronized ciliary movement can be identified by long, parallel trajectories (arrows), while small circular trajectories (arrowheads) indicate uncoupled ciliary movement from a single cell. Scale bar = 200 µm. F: distribution of average transport velocities of the microspheres. Only those microspheres that moved were included.
striction, and it has been demonstrated in vitro that mechanical compressive stress itself, at physiologically relevant levels, can activate the epithelium in asthmatic-like ways and induce airway wall remodeling events in the absence of inflammatory mediators (11, 12, 52, 60, 67, 68).

In this study, we showed that mechanical strain associated with bronchoconstriction, namely slow, dynamic lateral compression, can itself enhance viral infection of the cells in the airway mucosa. We suggest that such compressive strains are likely to increase transepithelial and subepithelial transport due to very small convective forces, as if squeezing a very dense sponge slowly. In turn, these small flows can drastically enhance transport of viruses, which are on the order of 100 nm and thus have a Stokes-Einstein diffusivity of $\sim 6 \times 10^{-8}$ cm$^2$/s, and other large particles. In fact, even a very small dynamic strain of 15% at 0.1 Hz should drive fluid movement on the order of 600 $\mu$m/s, making the contribution of convection orders of magnitude greater than that of diffusion in overall transport from the apical surface towards the basal end of the model. In addition, our data suggest that dynamic compression may also decrease the barrier function of the epithelium by inducing partial loss of tight junctions. Our finding that such small stresses could, indeed, enhance viral infection rate may have important implications for designing strategies to delivering viral-based therapies to the airways, e.g., in the treatment of diseases such as cystic fibrosis.
Furthermore, we observed particle trajectories that extended physiological ciliogenesis, both in length and percent coverage. The culture conditions presented here showed more medium composition for ciliogenesis and cultured for 21 days confluence (3, 15, 18, 49, 65). We further optimized the culture with fibroblasts within a 3D matrix (1, 11, 50, 51, 63, 64, 75), and culture in ALI after epithelial cells have reached 75%, the addition of specific factors to a serum-free media (15, 27, 55), and culture in ALI after epithelial cells have reached confluence (3, 15, 18, 49, 65). We further optimized the medium composition for ciliogenesis and cultured for 21 days at ALI. The culture conditions presented here showed more physiological ciliogenesis, both in length and percent coverage. Furthermore, we observed particle trajectories that extended across several cells, suggesting some degree of coordination between cells. However, we observed a high variability both in the transport velocities and in the type of motion of particles on the epithelium. In actual bronchi, cilia sweep upward to remove debris from the lungs, but since our model was symmetric in the x and y directions (without strain) we did not expect an overall directionality. The effects of dynamic strain on ciliary motion were not considered in this work, although it has been previously shown that mechanical stimulation leads to intra- and intercellular Ca²⁺ signaling that would increase ciliary beat frequency (5, 56, 66). Therefore, the increase in overall infection rate that we see is unlikely to be due to any changes in ciliary beat frequency or coordination. Instead, considering that mechanical compression causes buckling of the epithelium and mucosal folding, a virus could accumulate in the folds and transmigrate through the areas of damaged epithelium with an increased rate due to epithelial damage strain-enhanced transport.

To ponder the physiological relevance of the strain we applied to our in vitro models, we first considered that the strain magnitude within any bronchoconstricted airway wall is highly nonuniform; SMCs on the outer perimeter shorten to cause compression of the airway wall (25), but since the internal perimeter remains constant (maintained by the basement membrane), the epithelium buckles into the lumen (34, 40, 58, 74). Furthermore, strain varies radially from the epithelium to the subepithelium due to geometry, buckling, and different mechanical properties of epithelial vs. subepithelial regions. Finally, strain profiles are different in bronchoconstricted airways of normal vs. asthmatic patients, because of the mechanics and increased thickness of the remodeled airway wall (35, 53, 74) as well as increased contractile forces of asthmatic SMCs (8, 25, 57). A number of studies (29, 30, 32, 34, 47, 58) have shown that in models of induced (e.g., by methacholine or histamine) bronchoconstriction, the maximum level of SMC shortening (i.e., that which causes complete closure of the lumen) is 30–40%. These maximal levels of SMC or outer wall strain were induced in normal subjects, whereas asthmatic airways have thicker and stiffer lamina propria and submucosal layers; we therefore expect maximum compressive strains (but not necessarily stresses) in the asthmatic airway wall to be considerably <30%. Thus, it is reasonable to assume that 15% strain applied laterally to a rectangular model of the airway wall is a reasonable mimic of in vivo bronchoconstriction. As to the strain rate, bronchoconstriction occurs intermittently in vivo and can last from seconds to minutes (43) and thus our applied rate of 0.1 Hz was used to test the effects of a relatively slow compression and relaxation of the airway all model on a relevant timescale.

The model virus we used constitutes a well-characterized lentiviral system that is relatively easy to track, since the virus itself is not fluorescent but an infected cell will become fluorescent after expressing the GFP transgene. The lentiviral system that is relatively easy to track, since the virus itself is not fluorescent but an infected cell will become fluorescent after expressing the GFP transgene. The transduction efficiency of target cells depends partially on the type of glycoprotein used to coat retroviral vectors, since this influences the mechanism by which the virus enters the cell. The envelope we used to coat the virus is widely used due to its efficiency in entering most cell types: its receptor is thought to be a lipid component of the cell plasma membrane (LBPA) that is widely distributed on the surface of most cell types (69), and it is thought to enter the cell by receptor-mediated endocytosis.
It is commonly believed that a lentivirus with a VSV-G envelope most efficiently enters from the basal side of airway epithelial cells, although some studies (6, 37–39, 59, 72) show efficient apical infection. Since we introduced lentivirus originally on the apical surface of the epithelium, we can hypothesize that, in our model, mechanical stress either enhances the expression of the VSV-G receptor on the apical side or it enhances the access of the virus to the basal side through epithelial damage and/or enhanced transepithelial transport.

The fact that subepithelial fibroblasts were more frequently infected under mechanical stress suggests that convection played an important role, particularly considering that virus transport is critical for cell infection (since in this system an infected cell cannot infect another cell; only the free virus can infect another cell).

In conclusion, using an in vitro model of the airway wall with relevant epithelial barrier functions, we showed that cell infection by lentivirus applied on the apical surface of the mucosa is strongly enhanced by dynamic compressive strain of 15% at 0.1 Hz. The strain increased both the percentage of airway epithelial cells infected as well as that of subepithelial fibroblasts. This not only highlights the mechanical environment of the airways as an important factor in viral infection susceptibility but also suggests that dynamic mechanical stress may be an important design strategy in viral therapies aimed at delivering transgenes to cells of the airway wall.

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REFERENCES


