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Local influence of cell viability on stretch-induced permeability of alveolar epithelial cell monolayers

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Abstract

Ventilator induced lung injury (VILI), often attributed to over-distension of the alveolar epithelial cell layer, can trigger loss of barrier function. Alveolar epithelial cell monolayers can be used as an idealized in vitro model of the pulmonary epithelium, with cell death and tight junction disruption and permeability employed to estimate stretch-induced changes in barrier function. We adapted a method published for vascular endothelial permeability, compare its sensitivity with our previously published method, and determine the relationship between breaches in barrier properties after stretch and regions of cell death. After 4–5 days in culture, primary rat alveolar epithelial cells seeded on plasma treated polydimethylsiloxane membrane coated with biotin-labeled fibronectin, or fibronectin alone were stretched in the presence of FITC-tagged streptavidin (biotin-labeled membrane) or BODIPY-ouabain. We found that the FITC-labeling method was a more sensitive indicator of permeability disruption, with significantly larger positively stained areas visible in the presence of stretch and with ATP production inhibitor Antimycin-A. Triple-stained images with Hoescht (nuclei), Ethidium Homodimer (EthD, damaged cell nuclei) and FITC (permeable regions) were used to determine that within permeable regions intact cells were positioned closer to damaged cells than in non-permeable regions. We concluded that local cell death may be an important contributor to barrier integrity.

Keywords

tight junction; barrier properties; lung; acute lung injury; pulmonary transport

Introduction

Mechanical ventilation with large tidal volumes may produce over-distension of alveolar epithelium, resulting in ventilator induced lung injury (VILI) and barrier dysfunction associated with increased protein in the alveolar space, which increases osmotic transport of

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Conflict of Interest Statement

The authors (Song, Davis, Lawrence, and Margulies) have no conflicts of interest to disclose.

Ethical Standards

No human studies were conducted for this research. The animal was protocol approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC).

interstitial fluid into the airspace, lung wet weight, and permeability (17, 21, 31, 33). Similarly, in vitro models of mechanical ventilation, whereby cell monolayers are stretched at physiologically relevant magnitudes, have revealed that large stretch levels increase permeability, typically measured as a flux of radio-labeled or fluorescently tagged macromolecules from apical to basal sides of the cell monolayer (18, 19, 30) or transport of charged tracers measured via increased transepithelial/endothelial electrical resistance (TER) across the cell monolayer (9, 12, 26, 28). Given the paucity of permeable and distensible cell monolayer substrate materials, recently we reported permeability measurement whereby BODIPY-tagged ouabain (BO) in the apical media is able to bind to basolateral Na⁺-K⁺ ATPase pumps on epithelial cells that are only accessible when tight junction barrier properties are compromised (Figure 1A) (8, 11, 12, 14-16, 22, 37). This method is specific to epithelial cells, and is sensitive to the stretch-induced variation in the number of Na⁺-K⁺ ATPase pumps on the cell membrane (14). Beginning with a novel method for endothelial cells, we present a modified, highly sensitive method to measure transport of FITC-tagged avidin from the apical medium that binds to a biotin-labeled collagen coating on an impermeable monolayer substrate only when epithelial barrier function is disrupted (Figure 1B) (18). Previously we reported that large stretch magnitudes may compromise regional barrier integrity via disruption and reorganization of local tight junctions (TJs) between intact or damaged cells in the epithelial cell monolayer, and by formation of denuded regions as cells dissociated from the stretchable membrane (7, 8, 11, 15). We use this new FITC-tagging method to test our hypothesis that after stretch, regions with increased permeability are spatially related to the location of damaged adherent cells, rather than to locations of intact cells with compromised tight junctional integrity or as a result of the formation of large cell-free zones in the alveolar epithelial cell monolayers.

Materials and Methods

Isolation and culture of rat alveolar epithelial cells (RAECs)

Type II alveolar epithelial cells were isolated from male Sprague-Dawley rats (250~400g, n=3-14/per group) as described previously (14-16), and seeded at a density of 10^6 cells/cm² on polydimethylsiloxane (PDMS) silastic membranes (Specialty Manufacturing, Inc) with one of two treatments: fibronectin coating or biotinylated fibronectin, 42 µg/ml in MEM, (Boehringer Mannheim Biochemicals, Indianapolis, IN). Fibronectin was biotinylated using EZ-Link Sulfo-NHS-LC-LC-Biotin, 230 nM, Thermo Scientific, Waltham, MA). After one hour of suspension, the biotinylated fibronectin (B-FN) was coated onto O₂-plasma (Technics, Technics Inc.) treated PDMS membranes. The O₂-plasma treatment generates negative charges to reduce hydrophobicity of the surface (23, 32), and is necessary to enhance the biotinylated fibronectin attachment to the membrane and promote cell adhesion (36). In addition, fibronectin was coated on untreated and treated membranes to identify the role of plasma treatment in stretch-induced barrier disruption, as measured using the BO method. After 4-5 days in culture conditions with MEM supplemented with 10% fetal bovine serum (FBS), primary RAECs exhibit Type I RAEC characteristics (2, 13) and form a monolayer with tight junction proteins (TJs) and selective permeability that mimics intact alveoli (5, 7, 10, 12).

Mechanical stretch of RAEC monolayers

Monolayer wells, randomly assigned to experimental groups (3 wells from each rat were used in every experimental group, and n=3-14 rats were included in each experimental group), were serum deprived in DMEM with 20mM HEPES for an hour prior to stretch studies isolate responses of the RAECs from any potential stretch-associated reactivity to cytokines, growth factors, and enzymes in serum. For those RAEC monolayers on fibronectin-coated (FN) membranes, BODIPY-ouabain (BO) (2 μ M; in DMEM+HEPES; Invitrogen, Carlsbad, CA) was added to the apical media to identify regions where BODIPY is bound to basolateral Na⁺-K⁺ ATPase pumps that are exposed when TJ dysfunction permits paracellular transport to the intercellular region (Fig.1A). In all biotinylated fibronectin-coated (B-FN) wells with RAECs, FITC-streptavidin (25 μ g/ml in DMEM +HEPES; Invitrogen, Carlsbad, CA), Ethidium Homodimer (EthD, 35 μ l/ml; Invitrogen, Carlsbad, CA) and Hoechst (0.5 μ l/ml; Invitrogen, Carlsbad, CA) was added to the apical media prior to stretch to co-localize FITC-stained regions with damaged cells and with adherent cell nuclei from intact and damaged cells (Fig. 1B).

Monolayers on each of the two PDMS coatings were assigned to one of 4 study groups: equibiaxially stretched in our custom-designed system (34) at 0% (unstretched controls), 12%, 25%, and 37% change in surface area (SA) for 10 min at 0.25 Hz (Fig.1C), equivalent to inflations from functional residual capacity to 64%, 86%, and 100% total lung capacity (34), respectively. In previous studies we reported that 10 minutes was sufficient to reorganize actin, alter TJ content and configuration, and cell-cell spacing, and permeability(7, 11, 14, 15, 35). As a positive control for TJ disruption, unstretched RAEC monolayers were treated with Antimycin-A (AMA, 10 μ M in DMEM, n= 3 rats), previously shown to cause ATP depletion and tight junction disruption (1, 4, 14), or designated as vehicle controls (VC) and provided with 1 μ l of DMSO per 1ml of DMEM+HEPES.

Quantification of monolayer permeability and cell death

After stretch or antimycin-A treatment, monolayers were rinsed three times with DMEM +HEPES. Three image fields per well were captured by epifluorescence microscope (Nikon, TE300, Melville, NY), maintaining all image capture settings for all wells generated from a single rodent subject used as a cell source. Using a customized analysis program (Matlab, Mathworks, Natick, MA), the background FITC or BODIPY intensity was defined as the average field intensity obtained from the unstretched vehicle wells. To quantify stretch-induced permeability, the fields from stretched wells or antimycin-A treated wells were analyzed and the percent of the field with FITC-streptavidin-biotin (FITC-SB) or BO values above the threshold was determined for each field, averaged across the three fields and wells in a group, to yield cohort sizes 3-14 rats per group which were compared across stretch conditions and coatings.

In the triple-labeled images from the B-FN coated wells, the number of damaged cells (EthD) were divided by the number of cell nuclei (Hoechst) in every field (ImageJ, NIH, Bethesda, MD) to determine the percent of damaged cells, and values from the 3 fields per well were averaged.

Colocalization of cell death and permeable monolayer regions

To evaluate our hypothesis that regions with damaged cells were also the permeable regions in the FITC-SB wells, we created a customized routine in Matlab to classify all intact cells as being located in permeable (FITC-positive) or impermeable (FITC-negative) locations, and determine the nearest-neighbor distance between intact cells and damaged cells. Shorter live-damaged cell distances in permeable regions than impermeable regions would confirm our hypothesis that damaged cells are preferentially associated with permeable regions. The routine had four components: identify cells; classify them as intact or damaged; designate them as residing in permeable or impermeable regions; define the nearest-neighbor distance between every intact cell and the closest damaged cell. The cell identification component consisted of using Hoechst images to distinguish cell nuclei from spurious noise. Briefly, because Hoechst was very nucleus-specific, the Hoechst positive detection threshold for each rat was defined as the average Hoechst intensity in the three unstretched images obtained for that rat. All pixels above this threshold were considered Hoechst-positive in the stretched cell images. Using ImageJ, clusters of pixels larger than $25\mu\text{m}^2$ were designated as “nuclei” because epithelial cell nuclei are typically $7\mu\text{m}$ in diameter ($38.4\mu\text{m}^2$)(3). The next step was to identify those nuclei considered damaged, by overlaying the EthD images and Hoechst images. EthD positive regions were identified using a similar threshold and cluster definition method, with one difference. Because EthD had lower specificity to the nucleus and higher background intensity, the threshold employed for identifying EthD positive nuclei was the mean of the EthD signal in the unstretched images plus one standard deviation. After overlaying EthD images and Hoechst images, we defined intact cell nuclei as Hoechst-only stained nuclei, and “damaged” nuclei as those with both Hoechst and EthD positive staining. Our third step was to classify cells as residing on permeable regions (FITC positive), or impermeable (FITC negative) of the monolayer. We created idealized cell boundaries by extending Hoechst-stained nuclei digitally radially outward from the nucleus by $1.3\mu\text{m}$, increasing pixel regions two to three-fold to capture some of the cytoplasmic area in a conservative manner. When these irregular “cell” boundaries overlapped, a commercial watershed image analysis routine (The MathWorks, Inc.) was employed to create separate cell regions, allocating that region to one cell or the other. These idealized cell maps were overlaid with the FITC images, and cells were designated as located in permeable or impermeable regions. Because monolayer barrier property integrity is determined at the tight junction rather than the nucleus, any portion of the cell could reside in a permeable region to be classified as permeable. Finally, in the fourth and final step, we determined the spacing between every intact cell centroid in a permeable zone and the centroid of its nearest damaged cell neighbor, and averaged the results for the image field. Similarly, we calculated the distance between every intact cell centroid in an impermeable zone and the centroid of its nearest damaged neighbor, and averaged for the image field.

Statistical analysis

Every experiment was performed on $n=3$ wells obtained from at least three animals, with 3 image field replicates obtained from every well, and wells within a rat averaged. Data is presented as the average across all rats, and error bars express the standard error of the mean (SEM). Stretch magnitude and method (FITC-SB and BO) were compared via a two-way

ANOVA followed by a Tukey's HSD post-hoc analysis. Similar methods were used to compare nearest-neighbor distances in the permeable and impermeable regions. One-way ANOVA and Tukey's HSD post-hoc analysis was applied to evaluate the influence of stretch on the number of damaged cells. For all analyses, significance was defined as $p < 0.05$.

Results

Bodipy-ouabain method vs. FITC-streptavidin-biotin method

Comparing the new FITC-SB method with plasma treatment (Fig. 2 black bars) and our previously published BO method without plasma treatment (Fig. 2 white bars) revealed that 37% SA cyclic stretch for 10 min significantly increased permeability above unstretched monolayers and lower stretch levels of 12% and 25% SA for both methods, as indicated by increased stained region area (Table S1; FITC-SB, $50.71 \pm 6.74\%$; BO $32.68 \pm 2.64\%$ without plasma treatment). Furthermore, using both methods, permeability produced by stretch at 37% SA was even larger than unstretched wells treated with Antimycin-A (AMA) to disrupt TJs. Because the FITC-SB area was significantly larger than BO area at 37% SA, and because only the FITC-SB method revealed increases in permeability with Antimycin-A which were significantly larger ($17.51 \pm 3.27\%$) than unstretched VC wells, we conclude that the FITC-SB method is more sensitive to TJ disruption than our previously published BO method.

O₂-plasma treatment effect

Our previously published BO method did not rely on O₂-plasma treatment. In contrast, the new FITC-SB method required O₂-plasma treatment of the PDMS membrane to create a uniform distribution of B-FN and enhance cell attachment (Fig. S1). Because the FITC-SB method was more sensitive than the BO method, we rationalized that the plasma treatment may have been responsible for the findings. Thus, we evaluated the contribution of plasma treatment to the TJ-function sensitivity of the BO method. We hypothesized that plasma treatment was a significant factor between the two methods, such that the BO method with O₂-plasma treatment would be more similar to the FITC-SB method. We compared permeability (stained areas) for the BO method with (Fig 2 gray bars) and without O₂-plasma treatment (Fig. 2 white bars), and the FITC-SB method (Fig. 2 black bars) for three conditions: 0% SA with VC, 0% SA with AMA, and 37% SA with VC. In contrast to our hypothesis, BO with O₂-plasma treatment did not reveal significant increases in permeability above 0% SA with VC for either the stretch or AMA treatment (i.e. no asterisks on gray bars in Fig. 2 or in Table S1). Given our evidence (Fig S1) that O₂-plasma treatment results in a more uniform fibronectin distribution, we speculate that this enhanced fibronectin distribution may contribute to a decreased exposure of the BO to the basolateral cell surface of monolayers on treated membranes. We conclude that, in contrast to our hypothesis, O₂-plasma treatment actually decreases the sensitivity of the BO method, resulting in more disparate results from the FITC-SB method. However, the O₂-plasma treatment produces the required hydrophilic conditions for the FITC-SB method, and induces a more even distribution of fluorescently tagged fibronectin under the monolayer (Fig. S1).

Cell death and local permeability

Stretch associated monolayer RAEC death for the FITC-SB method was consistent with our previously published values (Fig 3A red-stained nuclei, Fig 3B)(35), and AMA treatment was not associated with increased cell death. Furthermore, Pearson's correlation analysis showed strong positive correlation (correlation coefficient =0.7908, $p<.0001$) between quantity of cell death and the amount of FITC-SB monolayer staining. In the triple-stained images, qualitatively we observed most damaged cells were in FITC-SB stained areas, especially at 37% SA with VC (Fig. 3A). We hypothesized that permeability increases related to the distribution of dead cells, not just the number or density of dead cells in a region. Therefore, our hypothesis was that the proximity of living cells to dead cells would be greater in permeable regions (i.e. distances would be smaller). To evaluate our hypothesis quantitatively, we evaluated the calculated the distance between each live cell and their closest damaged cell neighbor, and compared average "closest neighbor" distance calculated for the permeable (FITC-positive, Fig 3C black bars) regions to those in the non-permeable (FITC-negative, Fig 3C white bars) regions. The calculated minimum neighbor distance between live and damaged cells was significantly shorter in the permeable regions than the impermeable regions for each experimental condition, except 25% SA (Fig.3C). Therefore, we conclude that permeable, or FITC-SB staining, is characterized by live cells in close proximity to damaged cells (even in unstretched monolayers), compared to the impermeable regions.

Discussion

We have modified a method previously developed to measure permeability of endothelial cells plated on gelatin coated membranes (18) for use in pulmonary epithelial cells plated on fibronectin. In the modified FITC-SB method, we introduced O₂-plasma treatment to ensure a uniform distribution of the fibronectin coating and enhance cell attachment, and used sulfo-NHS-LC-LC-biotin instead of the NHS-LC-LC-biotin used previously (18) to reduce the uptake of biotin by the cells. Moreover, we compared the sensitivity of the FITC-SB method to our previously published method using BO (6), and noted several important advantages of the new method. The first advantage is that the FITC-SB method is not affected by stretch. Specifically, BO binds to the Na⁺-K⁺ ATPase pumps on the basolateral surfaces of the RAECs, and we have documented that stretch increases the number of pumps at the membrane and/or pump activity (14, 20), which increases the potential for BO staining regardless of TJ integrity. Second, the FITC-SB method has a superior mechanistic advantage over the BO method. As the tight junction is disrupted during mechanical stretch (7, 11), intercellular gaps form (Fig. 4), increasing opportunities for BO and FITC-SB to bind, and creating a fluorescently stained region where barrier properties are compromised. However, BO binds to the exposed basolateral cell surfaces but not to cell-free denuded membrane regions, likely underestimating the actual permeable region in the monolayer. In contrast the FITC-SB binds only the exposed biotinylated membrane between cells and in cell-free regions (Fig. 4). Taken together, the FITC-SB method presented in this report is a more sensitive and specific method to detect barrier permeability due to TJ failure, cell rupture, and cell removal than the BO method.

We determined that RAECs located in permeable FITC-stained regions were closer to damaged cells than those in intact regions of the monolayer, and hypothesize that there is a link between locations of cell death and barrier property disruption. We speculate that this association may be due to a stiffness discontinuity in the monolayer between intact and damaged cells (27) creating stress concentrations that affect the local mechanical integrity of the monolayer and/or due to paracellular and intercellular signals initiated in the damaged and dying cells (24, 25, 29) that influence the biochemical equilibrium of the intact cells nearby.

In conclusion, we have introduced a more sensitive and specific method to assess stretch-induced permeability of epithelial cell monolayers, and have used the method to show that damaged cells play important roles in local disruption of barrier in epithelial cell monolayers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1. Comparative schematic of permeability assessments of stretched monolayers
 A) With tight junction TJ disruption, BODIPY-tagged ouabain (BO) has access to cell basolateral surfaces and binds to Na⁺-K⁺ ATPase pumps located on the basolateral membranes of rat alveolar epithelial cells (RAECs). B) When TJ integrity fails or cell-membrane contacts rupture, FITC-tagged streptavidin binds to the biotinylated fibronectin-coated PDMS membrane. C) Custom-designed cyclic stretch device expands RAEC monolayers seeded on fibronectin-coated PDMS membranes, creating a uniform, equibiaxial deformation field (34).

Figure 2. Assessment method, stretch magnitude and membrane treatment alter monolayer permeability measurement

A. Representative micrographs of BODIPY-ouabain (BO) and FITC-Streptavidin-Biotin (FITC-SB) stained (green) areas in stretched (10 min, 37% Δ SA) and unstretched RAEC monolayers with and without AMA treatment. Bar =100 μ m. B. These images were enhanced after analysis to enhance visibility for publication. Permeable area, as a percent of the total field, comparing FITC-SB (n=5 with O₂ plasma treatment), BO with (n=3~5) and without (n=5~14) plasma treatment for stretch at 0%, 12%, 25%, 37% change in surface area (Δ SA), and with AMA in unstretched monolayers. Mean $\pm$ SEM. Asterisk (*) indicates

significant difference from 0% SA with VC in each method; Dagger (†) indicates significant differences from 12%, 25% SA with VC and 0% SA with AMA; and bars indicates significant differences between methods in each experimental group.

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Figure 3. Monolayer cell death rates and nearest-neighbor distances between live cells and damaged cells

A. Representative triple stained RAEC monolayers after 10 min of cyclic stretch or AMA treatment. Streptavidin-biotin stained permeable regions (FITC, green), nuclei of damaged cells (red, EtHD), and nuclei of all cells (blue, Hoescht) reveal higher stretch magnitudes results in more permeability and cell death. Bar = 100 μ m. B. Percent damaged cells (relative to total number of adherent cells in the field) in stretched and AMA treated monolayers (n=5/group). C. Distance from live cells residing in permeable and non-permeable regions to nearest neighbor damaged cells. All values, mean $\pm$ SEM. Asterisk (*) indicates significant difference from 0% Δ SA with VC; and bars indicates significant differences between groups. These images were enhanced after analysis to enhance visibility for publication.