Stiffness and heterogeneity of the pulmonary endothelial glyocalyx measured by atomic force microscopy

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O’Callaghan R, Job KM, Dull RO, Hlady V. Stiffness and heterogeneity of the pulmonary endothelial glyocalyx measured by atomic force microscopy. Am J Physiol Lung Cell Mol Physiol 301: L353–L360, 2011. First published June 24, 2011; doi:10.1152/ajplung.00342.2010.—The mechanical properties of endothelial glyocalyx were studied using atomic force microscopy with a silica bead (diameter 18 μm) serving as an indenter. Even at indentations of several hundred nanometers, the bead exerted very low compressive pressures on the bovine lung microvascular endothelial cell (BLMVEC) glyocalyx and allowed for an averaging of stiffness in the bead-cell contact area. The elastic modulus of BLMVEC glyocalyx was determined as a pointwise function of the indentation depth before and after enzymatic degradation of specific glyocalyx components. The modulus-indentation depth profiles showed the cells becoming progressively stiffer with increased indentation. Three different enzymes were used: heparinas III and I and hyaluronidase. The main effects of heparinase III and hyaluronidase enzymes were that the elastic modulus in the cell junction regions increased more rapidly with the indentation than in BLMVEC controls, and that the effective thickness of glyocalyx was reduced. Cytochalasin D abolished the modulus increase with the indentation. The confocal profiling of heparan sulfate and hyaluronan with atomic force microscopy indentation data demonstrated marked heterogeneity of the glyocalyx composition between cell junctions and nuclear regions.

atomic force microscopy; bovine lung microvascular endothelial cell; heparan sulfate; hyaluronan

The endothelial glyocalyx is a polysaccharide-protein coating on the luminal surface of the vascular endothelium and forms a negatively charged, complex meshwork. The primary glycosaminoglycan (GAG) constituents of glyocalyx are heparan sulfates (HS), chondroitin sulfates, and hyaluronan (HA). The syndecan family of transmembrane proteoglycans and membrane-bound glypicans both carry HS and chondroitin sulfate side chains (29, 33), while HA is a nonsulfated GAG. HA components and measure diffusion and the dynamics of albumin association within the glyocalyx expressed by bovine lung microvascular endothelial cells (BLMVECs) in vitro (31). Here, atomic force microscopy (AFM) was used to quantify the elastic properties of BLMVEC glyocalyx before and after enzymatic degradations of these components.

AFM has been a method of choice to measure mechanical stiffness of endothelial cells using indentation techniques (8, 24, 26, 28, 30). Typical AFM indentation experiments involve the use of a sharp tip that indents the cell membrane and exerts pressure on the membrane and cytoskeleton. Using AFM, Mathur et al. (24) have found fivefold differences in elastic moduli measured over the nucleus vs. the peripheral cell body of human umbilical vein endothelial cells. Ohashi et al. (26) have used AFM and finite-element analysis to show the increase in elastic moduli for bovine endothelial cells exposed to shear stress. Costa et al. (8) used non-Hertzian pointwise approach to analyze how the elastic moduli of human aortic endothelial cells changes with indentation depth (8).

We approached the measurement of glyocalyx stiffness using the same pointwise approach with one major difference: because the sharp AFM tip (typical r = 10 nm) could easily exert high local pressure on glyocalyx elements, or poke through the glyocalyx layer with little resistance, we substituted this sharp AFM tip with a larger silica bead (diameter 18 μm). Thus we traded the high resolution of sharp tip AFM for low compressive pressures and spatial averaging of mechanical properties in the bead-cell contact region. These lower compressive pressures and spatial averaging in the bead-cell contact area allowed us to determine the elastic modulus of BLMVEC in a pointwise fashion, as a function of the 8, before and after enzymatic degradation of specific glyocalyx components. Three enzymes were used: heparinase III (HSase III) or heparinase I (HSase I), both at the concentrations of 15

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mU/ml, or hyaluronidase (HAase) at 1.2, 12, and 50 U/ml concentrations. In addition, cytochalasin D was used to disrupt the cell cytoskeleton and differentiate between the elastic contributions of glycocalyx and underlying cellular structures.

METHODS

Cell culture. BLMVECs (Vec Technologies, Rensselaer, NY) were cultured onto glass coverslips (1 in. round, 0.17 mm thick, Fisher Scientific, Pittsburgh, PA) precoated with 0.4% bovine gelatin (Sigma-Aldrich, St. Louis, MO) for 1 h, followed by 100 μg/ml bovine fibronectin (Sigma-Aldrich, St. Louis, MO) for 1 h at 37°C and 5% CO2. BLMVECs were plated at a density of 2.5 × 10^5 cells/cm² and cultured for 7–10 days.

Enzymatic degradation of the glycocalyx. Glycocalyx components were selectively digested by incubating cells with HAase (from strep. hyalurolyticus, Sigma-Aldrich, St. Louis, MO; EC 4.2.2.1; concentrations 1.2, 12, or 50 U/ml), HSase I (Sigma-Aldrich; EC 4.2.2.7; 15 mU/ml), or HSase III (Sigma-Aldrich; EC 4.2.2.8; 15 mU/ml) in MCDB-131 medium supplemented with 25 mM HEPES, pH 7.4, 0.01% penicillin/streptomycin, and 1% BSA (Fraction V, Sigma) at 37°C and 5% CO2 for 1 h. Cells were then rinsed with the same medium and used in AFM experiments.

Cytochalasin disruption of cytoskeleton. The effect of cytoskeleton disruption on elastic modulus was determined by incubating confluent monolayers of BLMVECs with 100 nM cytochalasin D (Sigma-Aldrich; EC 244–804-1) for 30 min at 37°C and performing subsequent AFM indentation as described below. Cytochalasin D was initially solubilized in DMSO, then diluted in cell medium to 100 nM (~300 μM DMSO or 0.02%). Identical cytochalasin treatment was conducted with BLMVEC monolayers pretreated for 1 h with 50 U/ml HAs.

AFM indentation. A borosilicate glass microsphere (diameter = 17.3 ± 1.4 μm; catalog no. 9020, Duke Scientific, Palo Alto, CA) was glued to the tip of a rectangular AFM cantilever (nominal spring constant = 0.03 N/m) and mounted to the z-piezo on an Explorer AFM head (Topometrix, Santa Clara, CA). The AFM scanner was placed above the BLMVEC monolayer covered with medium, and the bead was brought into contact with cells. The loading force was minimized to prevent any cell damage. In the indentation measurement, the cantilever deflection was measured by a position-sensitive diode (PSD) as a function of z-piezo displacement producing raw AFM data. The measurements were taken at multiple (n > 80) locations on the BLMVEC monolayer surface. The loading rate was 10 μm/s, and the maximal loading force varied between 5 and 10 nN. The typical δ was up to ~500 nm. After the indentation measurements were completed, the sample topography was mapped by scanning the same AFM bead over the BLMVEC monolayer over a (100 μm)^2 region. From the topological scans, the specific loci of cell-cell junctions and cell nuclei were judiciously assigned to each indentation run (Fig. 1). The indentation runs that did not localize exactly to either of the two locations were excluded from the analysis. The contribution of individual glycocalyx components to the overall stiffness was assessed by the enzymatic digestions, followed by subsequent AFM indentation measurements.

Data analysis. The AFM indentation data were analyzed by finding the point of contact between the bead and the cell surface layer in raw AFM data and then performing a pointwise analysis to determine the stiffness of the glycocalyx as a function of the δ. The stiffness of the cantilever (in N/m) was calibrated using the AFM instrument built-in software function, which also provided the conversion factor between the cantilever deflection signal (in nA) and cantilever force (in nN). Once the stiffness of the cantilever was known, a hypothetical rigid substrate deflection-displacement line, with the slope equal to the negative cantilever stiffness, was plotted through the contact point, as shown in Fig. 2A. The indentation into the glycocalyx, δ, was found by subtracting the displacement value of each data point from the displacement value on the rigid substrate line at the identical force, F. This procedure produced the force-indentation F(δ) curve shown in Fig. 2B. To account for spherical geometry of the AFM probe, the indenter geometry function for a sphere of radius R was used (3):

\[ \Phi(\delta) = \frac{4}{3\pi} \sqrt{R^3 - \delta^3} \]  

where R is that of the bead indenter. This indenter geometry function was then used to find the pointwise elastic modulus, E, for every data point on the F(δ) curve (8):

\[ E(\delta) = \frac{F(\delta)}{2m\Phi(\delta)} \]  

The E of a cell (in kPa), plotted as a function of indentation, δ (Fig. 2C), showed that the E of control BLMVECs glycocalyx was constant up to the δ values of ~200 nm. The depth of indentation, where the E(δ) curve shows an inflection, was used as an estimate of the effective thickness of the glycocalyx, δ_e. Statistical analysis of BLMVEC moduli recorded before and after enzymatic treatments was performed using a Wilcoxon-Mann-Whitney rank-sum test.

Confocal imaging. BLMVEC monolayers were treated with enzmies, washed with phosphate-buffered saline, and fixed with 4% paraformaldehyde at room temperature. HS was immunostained with anti-HS (HEpSS-1, US Biologicals, Swampscott, MA) and incubated with Alexa Fluor 596 labeled anti-IgM-k (BD Biosciences, San Jose, CA). HA was localized using biotinylated HA-binding protein (US Biologicals; H7980–35) and then labeled with avidin-Alexa Fluor 488 conjugate (Invitrogen, Carlsbad, CA; A-21370). A FV1000-XY, Olympus IX81 confocal microscope and a 60 × NA 1.45 oil immersion lens were utilized for imaging (Core Facilities, University of Utah). Images of (200 μm)^2 area were taken with vertical separation distance of 0.2 μm. The fluorescence intensity profiles through the confocal images stack were extracted using ImageJ software (W.
RESULTS

Analysis of AFM spherical bead indentation experiments yielded two dependent variables: $E$ and $\delta$. Figure 3 shows how average modulus changes with the $\delta$, $<E>(\delta)$ at cell junctions (Fig. 3A) and nuclei (Fig. 3B), before and after enzymatic digestions with HSase III (15 mU/ml) and HAase (50 U/ml). The difference between BLMVEC controls and the enzymetreated cells was remarkable; in the case of the controls, the $E$ initially rose slowly with indentation and reached $\sim 1$ kPa at $\delta = 600$ nm at the junctions. After the enzyme digestion, the modulus increased more rapidly at smaller indentations for both enzymes and also showed signs of leveling at large indentations. The modulus of control BLMVECs had a low value of $\sim 0.25$ kPa and was approximately constant up to the $\delta$ values of $\sim 200$ nm. Upon further indentation, the modulus increased, which indicated that the loading forces compressing the glycocalyx were progressively transmitted to the less compliant cell membrane and underlying cytoskeleton. The finding that the enzymatic treatment increased stiffness in the region between $0 < \delta < 600$ nm relative to controls indicated that the glycocalyx, which resided in this region, was being degraded. For nuclear locations, however, the difference between $<E>(\delta)$ data for controls and enzyme-treated BLMVECs was indistinguishable. The average modulus diverged only at larger $\delta$ values, and HSase III action made the cells stiffer, while HAase made them a bit softer (Fig. 3B).

One way to analyze the $<E>(\delta)$ data is by using two-layer composite compliance model (10):

$$
\frac{1}{E(\delta)} = \frac{1}{E_{\text{glycocalyx}}} \left( e^{-\alpha \delta / \delta_g} \right) + \frac{1}{E_{\text{cell}}} \left( 1 - e^{-\alpha \delta / \delta_g} \right)
$$

where $E_{\text{glycocalyx}}$ and $E_{\text{cell}}$ are the mean elastic moduli of the glycocalyx and the cell, respectively, and $\alpha$ is a parameter defining the mechanical interlayer interactions. The compliance of the spherical bead indenter was assumed to be much smaller compared with the other two right-hand side terms and was omitted from analysis. The model accounts for the transfer of mechanical deformation between the two layers, i.e., the glycocalyx and the cell, by the term $\exp(-\alpha \delta / \delta_g)$ (20). This transfer function is dependent on the $\delta$, the $\delta_g$, and the extent of interlayer interactions, which are dependent on the local composition of glycocalyx. A fit to the two-layer composite compliance model for BLMVECs treated by HSase III and HAase yielded the best fitted parameters as follows: $E_{\text{cell}} = 2.93 \pm 0.38$ kPa and $E_{\text{glycocalyx}} = 0.26 \pm 0.03$ kPa for HSase III, and $E_{\text{cell}} = 2.35 \pm 0.31$ kPa and $E_{\text{glycocalyx}} = 0.28 \pm 0.03$ kPa for HAase (50 U/ml). The $\delta_g$ was estimated to be 420 nm (HSase III) and 450 nm (HSase 50). The fitted $\alpha$ parameter was $\sim 2.2$; however, because it appears in the exponent ratio $\alpha / \delta_g$, its effect on the fit was strongly affected by estimate of the $\delta_g$. The $<E>(\delta)$ results for untreated BLMVECs did not display a sigmoidal shape, so the fitted results were inconclusive.

The biomechanical role of cellular structures below the glycocalyx was investigated by treating the cells with cytochalasin D. Cytochalasin D is known to inhibit actin polymerization within the cell, thus causing softening of the cell (18). Figure 4 compares the $<E>(\delta)$ data at the cell junctions for untreated BLMVECs, cells treated with cytochalasin D, and cells treated first with enzyme HAase (50 U/ml) and then with cytochalasin D. The $E$ of cytochalasin D-treated BLMVECs remained $<0.5$ kPa for the whole range of $\delta$ values ($\delta < 500$ nm). The pretreatment of cells with enzyme HAase followed by cytochalasin D treatment resulted in an $E$ profile that was almost indistinguishable from the cell treatment with cytochalasin D alone. A similar trend of cytochalasin D cell softening was found at the nuclear locations (data not shown). The results of the cytochalasin D experiments confirmed that the observed increases in elastic moduli at intermediate indenta-

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Fig. 2. Conversion of raw AFM data into the pointwise elastic modulus $E$. $A$: typical raw AFM data for untreated BLMVEC and rigid samples. $B$: BLMVEC force vs. indentation data. $C$: the pointwise $E$ calculated from the force-indentation data plotted as a function of indentation depth $\delta$.

Rasband, National Institutes of Health) at 6 $\mu$m$^2$ areas at junction and nucleus locations.
tions (100 < δ < 500 nm), for control and enzyme-treated BLMVECs (Figs. 3 and 4), were due to the progressive transmission of the compressive loads from the glycocalyx to the underlying cytoskeletal structure. Consequently, the effective stiffness of glycocalyx was represented by the modulus at 100-nm δ, $E_{100}$. Figure 5 shows the actual $E_{100}$ data for each indentation run and for all enzyme treatments measured at cell junctions. Figure 5A compares the digestion of BLMVEC glycocalyx with HSase III (15 mU/ml) or HAase (50 U/ml) to the control. For both enzymes, the mean elastic moduli $E_{100}$ (shown by the horizontal lines), as well as the spread of $E_{100}$ data, increased upon the enzyme digestion (HSase III, $P = 0.003$, HAase, $P = 0.035$, each compared with the controls). Clearly, the enzymatic digestion made the BLMVECs appear a bit stiffer compared with controls. The majority of the $E_{100}$ data, however, remained <0.4 kPa, indicating the enzymes might not have completely digested the glycocalyx. Figure 5B compares the effect of HAase concentrations on $E_{100}$. Similar findings, such as larger spread of moduli and increases in $<E_{100}>$, were observed for BLMVECs treated with HAase at low (1.2 U/ml, $P = 0.017$) and high (50 U/ml, $P = 0.035$) concentration compared with controls. Figure 5C compares two enzymes that are known to degrade HS: HSase I and HSase III. While the repeated treatment of BLMVECs with HSase I showed very similar $<E_{100}>$, only the treatment with HSase III showed significant difference from the controls [HSase I (first run) $P = 0.192$, HSase I (second run) $P = 0.100$, HSase III, $P = 0.050$].

Confocal imaging of enzyme-treated and control BLMVECs was used to create the concentration profiles for HS (Fig. 6) and HA (Fig. 7) at both cell junction and nuclear locations. For untreated BLMVECs, a higher concentration of HS was found at the cell junctions compared with nuclear locations (Fig. 6, A vs. B). The action of HSase III (15 mU/ml) reduced the integrated fluorescence intensity at both locations (junctions: a 65% decrease, nuclei: a 54% decrease) and shifted the fluorescence maxima to smaller distances from the basal side of the cells. This indicated the enzyme was more effective at the upper cell surface, as expected.
The confocal imaging also revealed that the spatial distribution of HA was different from HS; the fluorescence intensity was much larger above the nuclei than at the cell junctions (Fig. 7, A vs. B). The HAase (50 U/ml) digestion of HA was more effective at nuclear locations, reducing the integrated fluorescence intensity by 58%, than at the cell junctions (a mere 32% reduction). Like in the case of HS digestion, the maxima of the HA fluorescence intensity shifted to smaller distances from the basal side of the cells upon the treatment with HAase.

**DISCUSSION**

The goal of the present study was to characterize the effect of glycocalyx components on stiffness of BLMVEC glycocalyx and to elucidate the contributions of HS and HA. The technique selected was spherical probe AFM, because the probe exerted smaller loading forces over larger contact area than in the case of typical indentation with a sharp AFM tip. For example, the contact area of a spherical bead ($d/H_1 = 100$, $H_2 = 92$) at 50 nm indentation was $2.8 \, \mu m^2$, or a circular area with $r = 0.94 \, \mu m$, and the bead exerted only 180-Pa pressure at 0.5 nN load. The same spherical bead used to indent the cells was used to scan the topography of BLMVEC monolayers and to develop coordinates that allowed precise assignment of indentation locations at cell junctions, which are believed to be the loci of mechanotransduction (37). For comparison, indentation measurements were also carried out at the nuclear locations.

The force-indentation data, $F(\delta)$, were used in a pointwise calculation of the $E$ as a function of the $\delta$, $E(\delta)$ (8). A large number of $E(\delta)$ curves (typical $n > 30$) were averaged to analyze the difference between BLMVEC controls and cells treated with enzymes. In the case of controls, the $E(\delta)$ measured at the cell junctions was essentially flat up to 200-nm indentation and then reverted to an increasing function up to an indentation of 600 nm. After enzymatic digestion with HSase III (15 mU/ml) or HAase (50 U/ml), the average modulus $E(\delta)$ increased more rapidly at smaller indentations at $\sim 100$-nm $\delta$ (Fig. 3A). The effective $\delta_e$ was estimated from the inflection of $E(\delta)$ curves where possible, or from the model described by Eq. 3. As previously reported, the $\delta_e$ determined...
by in vivo fluorescence imaging of mesenteric vessels (or hamster cheek pouches) (11), by in vitro measurements on BLMVECs utilizing fluorescence correlation spectroscopy, and by confocal immunohistochemistry (31) was found to be larger than 500 nm. This thickness agrees with the estimates from individual $E(\delta)$ curves (for example, see Fig. 2C). The average $<E>(\delta)$ data after HSase III or HAsse digestion showed that the enzymes decreased this value to $\sim$420–450 nm. The two-layer composite compliance model (10) was used to assess the biomechanical properties of glycocalyx and underlying cell membrane and cytoskeleton. The fit to the model yielded reasonable $E$ estimates for glycocalyx and underlying cellular structures, but was less sensitive to the $\delta_g$ and the parameter $\alpha$. Such simple models do not represent the physical properties of cellular structures well; neither glycocalyx nor underlying cytoskeleton is a uniform homogeneous layer, but is instead a mesh of interconnected stiffer and softer elements (4). Hence, the usefulness of such model might be rather limited. In addition, not all $<E>(\delta)$ data could be fitted well with the model, because some $E(\delta)$ curves did not display sigmoidal shape, which was a prerequisite for a good fit. This was especially noticeable for BLMVEC controls (Fig. 7A). Larger variations of $\delta$ values to show the leveling of the $E$ (Fig. 3). One may infer in such cases that the $\delta_g$ was larger than the $\delta$ used. The estimates for the $\delta_g$ from the inflection of $<E>(\delta)$ confirm such an inference. The change of the glycocalyx composition, for example, by enzymatic digestion, has the potential to affect in such cases that the $\delta_g$ is a significant difference between $<E>(\delta)$ at cell junctions and at nuclear locations. One can speculate that the mechanical coupling between glycocalyx and the underlying cytoskeleton at the cell junctions is different than at the nuclear locations. Confocal profiling showed that nuclear locations of BLMVECs

![Confocal vertical profiles of HA before and after enzyme digestion with HA50 (n = 3). A: cell junction location. B: nuclear location. Negative controls were not treated with the enzyme or HA binding protein, but only stained with avidin-Alexa Fluor 488 conjugate. The insets show confocal images of untreated and HAsse-treated BLMVECs.](image-url)
were predominantly decorated with HA polymer chains (Fig. 7, A vs. B), known to be noncovalently attached to cell membrane receptors and to HS proteoglycans. In contrast, the cell junctions appeared to be richer in HS than the nuclear regions (Fig. 6, A vs. B). Admittedly, confocal vertical profiling does not have the same resolution as lateral confocal imaging. However, it was possible to resolve the vertical distribution of HS above the nuclei where the stain showed two peaks, indicating that HS is present at both the basal and upper cell surfaces (Fig. 6B). No such resolution was possible for the HA stain. In general, the fluorescence was not eliminated by the enzyme treatments; the enzymes predominantly digested HA and HS polymers from the upper cell surface, which was exposed to enzyme solution. We conclude that the enzymes did not completely digest each glyocalyx component, as indicated by depth profiles (Figs. 6 and 7).

Two forms of HSases (HSase I and HSase III) have been tested for their effects on glyocalyx stiffness. HSase I cleaves disaccharide substrates that have a higher sulfate content, whereas HSase III cleaves unsulfated disaccharides. It has been reported that endothelial HS participate in both flow (13, 27) and pressure-induced (12) mechanotransduction that subsequently activates nitric oxide synthase. Increased levels of nitric oxide are associated with barrier dysfunction, as assessed by increased hydraulic conductivity (6, 12, 13). It has been reported that selective removal of cell-surface HS with HSase III abolished pressure and flow-mediated nitric oxide production and the associated barrier dysfunction, establishing a direct link between the glyocalyx and barrier-dependent mechanotransduction (12, 13, 27). According to the present results, only HSase III had a significant effect on increasing the glyocalyx modulus (Fig. 5C, P = 0.050), thus supporting the previous findings. HAase degradation of the glyocalyx at higher enzyme concentrations also resulted in a significant increase in E100 compared with untreated cells (Figs. 5, A and B, P = 0.035). It is, therefore, possible that HA acted as a softer, multiaattachment cross-linker within the glyocalyx structure, and that its removal exposed stiffer elements of the glyocalyx.

One major conclusion from the present study is that enzymatic digestion of a single GAG in the glyocalyx leaves the other components in place so that they are able to maintain a similar stiffness in the probe-cell contact area. For example, it is likely that digestion of HA, which was found more concentrated above nuclear regions, leaves HS and associated transmembrane proteins, such as syndecan core protein, to transmit the compressive forces exerted by the AFM probe. Similarly, digestion of HS, which was found more concentrated in the cell junction areas as well, leaves syndecans in place and possibly also associated HA polymer. Yet these spatial differences in the composition of vascular glyocalyx must exist for functional reasons, which have yet to be fully elucidated.

Summary. AFM was used to assess the mechanical properties of BLMVEC glyocalyx: its modulus and thickness. The AFM indenter was a silica bead (diameter ~18 μm) used instead of sharp AFM cantilever tip. This resulted in low compressive pressures on the glyocalyx, allowing determination of the E in a pointwise fashion as a function of the δ. The modulus-δ profiles showed cells becoming progressively stiffer with the indentation. Three different enzymes were used to digest glyocalyx components: HSases III and I and HAase. For HSase and HAase treatments, the E in the cell junction increased more rapidly at lower indentations than in controls. These enzymes also reduced the δ. Cytochalasin D abolished the modulus increases with the indentation. It was found that the digestion of a single glyocalyx component leaves the other components in place so that they were able to maintain a similar stiffness in the probe-cell contact area. More importantly, the combined confocal profiling and AFM results demonstrated marked heterogeneity of the glyocalyx spatial composition between cell junctions and nuclear regions.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

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