Terminal sialic acids are an important determinant of pulmonary endothelial barrier integrity

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Cioffi DL, Pandey S, Alvarez DF, Cioffi EA. Terminal sialic acids are an important determinant of pulmonary endothelial barrier integrity. Am J Physiol Lung Cell Mol Physiol 302: L1067–L1077, 2012. First published March 2, 2012; doi:10.1152/ajplung.00190.2011.—The surface of vascular endothelium bears a glyocalyx comprised, in part, of a complex mixture of oligosaccharide chains attached to cell-surface proteins and membrane lipids. Importantly, understanding of the structure and function of the endothelial glyocalyx is poorly understood. Preliminary studies have demonstrated structural differences in the glyocalyx of pulmonary artery endothelial cells compared with pulmonary microvascular endothelial cells. Herein we begin to probe in more detail structural and functional attributes of endothelial cell-surface carbohydrates. In this study we focus on the expression and function of sialic acids in pulmonary endothelium. We observed that, although pulmonary microvascular endothelial cells express similar amounts of total sialic acids as pulmonary artery endothelial cells, the nature of the sialic acid linkages differs between the two cell types such that pulmonary artery endothelial cells express both α(2,3)- and α(2,6)-linked sialic acids on the surface (i.e., superficially), whereas microvascular endothelial cells principally express α(2,3)-linked sialic acids. To determine whether sialic acids play a role in endothelial barrier function, cells were treated with neuraminidases to hydrolyze sialic acid moieties. Disruption of cell-cell and cell-matrix adhesions was observed following neuraminidase treatment, suggesting that terminal sialic acids promote endothelial barrier integrity. When we measured transendothelial resistance, differential responses of pulmonary artery and microvascular endothelial cells to neuraminidase from Clostridium perfringens suggest that the molecular architecture of the sialic acid glycomes differs between these two cell types. Collectively our observations reveal critical structural and functional differences of terminally linked sialic acids on the pulmonary endothelium.

glyocalyx; neuraminidase; lectin; α(2,3), α(2,6)

THE ENDOTHELİUM FORMS a semipermeable barrier between the blood and underlying tissue. The endothelial glyocalyx consists of glycoproteins, glycolipids, proteoglycans and glycosaminoglycans, which coat the cell surface (18, 23) (Figure 1A). The carbohydrate network that contributes to the glyocalyx is very complex, and, to date, the structure of these complex carbohydrates and their role in endothelial barrier function are poorly understood. Cell surface carbohydrates play a role in communication events such as microbial invasion, inflammation, and immune response; slight alterations in the patterns of glycosylation are known to cause dramatic changes in cellular behavior (33). In the pulmonary vasculature the glyocalyx of pulmonary artery endothelial cells (PAECs) exhibits differences compared with the glyocalyx of capillary (pulmonary microvascular) endothelial cells (PMVECs) (14). Additionally, PAECs and PMVECs exhibit distinct endothelial barrier properties, where PMVECs form a tighter barrier than PAECs (12, 22). It is currently unknown, however, whether overall glyocalyx structure plays a major role in determining the distinct barrier properties of PAECs and PMVECs in the pulmonary vasculature.

Two components of glyocalyx structure that may play a role in determining function in glycoproteins are 1) the terminal sugar residue(s) on the oligosaccharide chain(s) and 2) the amino acid residue attachment site (N- vs. O- for the oligosaccharide chain to the protein (5, 23). Sialic acid is a negatively charged carbohydrate moiety that is found in oligosaccharides, polysaccharides, glycoproteins, gangliosides and lipopolysaccharides (28). A distinguishing feature of sialic acid is that it is typically found at the distal ends of carbohydrate chains (33) (Fig. 1B). Sialic acids constitute a family of acidic nine-carbon keto sugars and include N-acetylmuramic acid (Neu5Ac), 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid (Kdn), 5,7-diamino-3,5,7,9-tetra-deoxy-D-glycero-D-galacto-nonulosonic acid (legionaminic acid), and 5,7-diamino-3,5,7,9-tetra-deoxy-L-glycero-L-manno-nonulosonic acid (pseudoaminic acid); the name sialic acids derives from their discovery in saliva submaxillary mucins (2). The sialic acid family is made of ~50 members (28). The structures of the most frequently occurring sialic acids in mammalian systems are Neu5Ac and Kdn (Fig. 1C). Sialic acids are found in a variety of glycosidic linkages, principally as α-2,3- or α-2,6-linkages to galactose or N-acetylgalactosamine residues in N- and O-linked glycans (Fig. 1D), although α-2,8-linked sialic acid oligomers can be found as well, for example in the neural cell adhesion molecule protein, a member of the immunoglobulin superfamily (25). Some other sialic modifications result from substitution of the hydroxyl groups present on carbons 4, 7, 8, and 9 by methyl, acetyl, lactyl, sulfate, or phosphate groups (32).

Sialic acids contribute significantly to the overall negative charge of glycoconjugates and cell surfaces (32). This negative charge contributes to the formation of a protective electrostatic shield, which has been shown to be important for antiadhesive repulsion between circulating cells and vessel walls (13, 27), as well as protection of parts of a tethered glycoprotein from proteolytic attack (33). Indeed, Görg and colleagues (8) determined that surface sialic acids provide protection of an intact endothelium against proteolysis. In addition to its protective role, sialic acid also serves to modulate physicochemical properties of specific proteins and lipids to which it is attached (27), influencing overall protein/lipid structure and function. As...
such, sialic acids can also play a role in cellular recognition and signaling events (21, 28, 34).

In the pulmonary endothelium, the roles of sialic acid are not well understood. In the present study, we quantitate sialic acids present in PAECs and PMVECs and utilize exoglycosidase enzymes and stereospecific fluorescent lectin binding to identify specific sialic acid configurations on the two cell types. Finally, we demonstrate the contribution of terminal sialic acids to endothelial barrier integrity.

MATERIALS AND METHODS

Materials. Sialic acid assay kit was purchased from BioVision Research Products (Mountain View, CA). Fluorescently tagged lectins were obtained from Vector Laboratories (Burlingame, CA), EY Laboratories (San Mateo, CA) or Sigma-Aldrich (St. Louis, MO). Hank’s Balanced Salt Solution (HBSS) was purchased from Invitrogen (Carlsbad, CA). Neuraminidases and other reagents were obtained from Sigma-Aldrich.

Animals. Protocols were approved by the Institutional Animal Care and Use Committee, conforming to the NIH Guide for the Care and Use of Laboratory Animals. Adult male CD rats (n = 3, Charles River, Wilmington, MA) were anesthetized with pentobarbital sodium (50 mg/kg body wt, intraperitoneally). After confirmation of anesthetic plane, a tracheostomy was performed, and the animals were ventilated at 6 ml/kg body wt (Inspira mechanical ventilator, Harvard Apparatus, Boston, MA), 55 strokes/min, room air. Following a thoracotomy, a PE240 plastic cannula was placed and secured in the pulmonary artery and the left atrium. After that, the lungs and the heart were excised en bloc and suspended in a force transducer for permeability assessment as described previously (1).

Pulmonary endothelial permeability assessment. Excised-suspended lungs were ventilated at 6 ml/kg body wt, 55 strokes/min, with a mixture of gases containing 5% CO2-21% O2, balanced with N2, and a positive end-expiratory pressure of 3 cm H2O. Perfusion was maintained at a constant flow (0.045 ml/g body wt) with Earle’s buffered solution containing 4% purified bovine serum albumin and calcium chloride adjusted at 5.5 mM. Total circulating volume was 30 ml. After vascular recruitment maneuvers, Kf was measured at baseline and after 30-min perfusion with 0.5 U/ml neuraminidase from Vibrio cholerae. Kf was calculated as an index of hydraulic permeability by measuring the filtration coefficient after raising venous pressure from 5 to 14 cm H2O for 15 min. The resulting weight gain during the last 2 min of raising the venous pressure was divided by the concomitant increment of capillary pressure, and values were normalized to predicted lung weight. After completion of the second Kf, venous pressure was returned to baseline for 5 min, and the lower right lobe was immersion fixed in buffered formalin. Lung specimens were embedded in paraffin, cut in 5-μm sections and stained with hematoxylin and eosin for light microscopy examination.

Cell culture and isolation. Rat PAECs and PMVECs were isolated and cultured as described previously (12, 14, 20) under the approval of the Animal Care and Use Committee of the University of South Alabama. Cultured cells were used between passages 5 and 20.

Sialic acid quantitation. Free sialic acids and total (free plus bound) sialic acids were quantified. For free sialic acid measurement, whole cell lysate samples were used; for total sialic acid measurement, hydrolyzed cell lysate samples were used.
Sialic acid hydrolysis. Whole cell lysate (20 μl) was combined with 80 μl of 0.05 N H₂SO₄ (hydrolysis reagent) and incubated at 80°C for 60 min. Samples were briefly centrifuged at 14,000 revolution/min (16,000 g), after which 20 μl of 1 M NaOH (neutralization reagent) was added and the mixture centrifuged again at 14,000 revolution/min. The supernatants were removed and used for total sialic acid quantification.

Sialic acid quantification. Sialic acid quantitation was carried out using the Sialid (NANA) Assay kit from Biovision (Mountain View, CA) following the manufacturer’s protocol. Neuraminidase from Clostridium perfringens was prepared at various concentrations in HBSS supplemented to 2–4 mM Ca²⁺ final concentration. Cultured monolayers of PAECs or PMVECs were washed twice with HBSS, following which neuraminidase solutions were added. Cells were incubated for 1–5 h (room temperature, 37°C or combination) with neuraminidase and were then washed twice with HBSS before microscopy or lectin binding. Cells were imaged on an Olympus IX70 inverted microscope using Spot software (Diagnostic Instruments, Sterling Heights, MI) or on a Nikon TE200 inverted microscope using Pixera Viewfinder software (Pixera, Santa Clara, CA).

Heat-inactivation of neuraminidases. Neuraminidases were inactivated by heating at 100°C (boiling water or heat block) for 30 min as was previously described (6).

Measurement of protease activity. Protease activity in neuraminidase preparations was measured using the Pierce Fluorescent Protease Assay Kit (Thermo Scientific, Rockford, IL) following manufacturer’s instructions. The standard curve was prepared using trypsin with the lowest concentration at 1 ng/ml.

Lectin binding assay. Lectin solutions were prepared at the following concentrations in HBSS: TRITC-tagged Arachis hypogaea agglutinin (10 μg/ml), FITC-tagged Maackia amurensis agglutinin (MAA, 20 μg/ml), Texas Red-tagged Sambucus nigra agglutinin (SNA, 20 μg/ml), or FITC-tagged SNA (10 μg/ml). PAEC and PMVEC monolayers were washed twice with HBSS, following which lectin solutions were added. Cells were incubated with lectins for 15–30 min at room temperature. They were then washed twice with HBSS and imaged.

Electric cell-substrate impedance sensing. Electric cell-substrate impedance sensing (ECIS) experiments were conducted using an Applied Biophysics Model 1600R instrument (Applied Biophysics, Troy, NY). Cells were grown to confluence in eight-well ECIS arrays. On the day of experiments, cells were washed twice with HBSS (supplemented to 4 mM Ca²⁺ final concentration), following which HBSS was added to each well. Resistance measurement was commenced, and a baseline value was obtained over at least 1 h. This was followed by the addition of neuraminidase solution or buffer control to each well and continued resistance measurement 4 (or more) h.

RESULTS

Sialic acids contribute to the glycocalyx of PAECs and PMVECs. We initially wished to determine whether sialic acids are expressed in the glycocalyx of PAECs and PMVECs. As such, we measured free and total sialic acid levels of both cell types, and, as expected, sialic acids were detected in both. Comparison of free and total sialic acids revealed that PAECs and PMVECs express similar levels (Fig. 2A). A second method of demonstrating the presence of sialic acids on the cell surface is to treat cells with a neuraminidase, i.e., sialidase, followed by staining with fluorescein-conjugated lectin from Arachis hypogaea. Neuraminidases are enzymes that cleave via hydrolysis α(2–3)−, α(2–6)−, and α(2−8)-linked terminal sialic acid residues bound to Gal, GlcNAc, GalNAc, AcNeu, or GlnNeu residues of oligosaccharides, glycolipids, and glycoproteins (17). Neuraminidases from different sources exhibit different specificities for sialic acid linkages hydrolyzed (4, 24). The lectin from Arachis hypogaea binds to the sequence Gal(β1,3)GalNAc, also known as T-antigen (19, 24). When the T-antigen sequence is sialylated, lectin from Arachis hypogaea does not bind to the disaccharide (10). However, as in the case of red blood cells, following treatment with neuraminidase, the T-antigen is exposed on the cell surface allowing the lectin to bind (19). Indeed, this approach has already been used to demonstrate loss of sialic acids from pulmonary endothelial cell surfaces (26). For these experiments, PAECs and PMVECs were treated with neuraminidase from Clostridium perfringens, which cleaves α(2−3), α(2−6), and α(2−8)-terminal sialic acid residues (3, 4, 17). The Arachis hypogaea lectin did not bind to control cells but exhibited strong binding to neuraminidase-treated cells as evidenced by positive fluorescence in treated cells (Fig. 2B), revealing the underlying Gal(β1,3)GalNAc epitope.

PAECs and PMVECs differ in their sialic acid configurations. PAECs and PMVECs display many phenotypic differences. For example, PMVECs have a shorter doubling time compared with PAECs (14), and they also tend to form a tighter endothelial barrier (12, 22). Therefore, we wanted to determine whether PAECs and PMVECs differ in their expression of sialic acids. One way in which sialic expression can differ is in quantity; however, the sialic acid levels did not differ significantly between PAECs and PMVECs. Other differences in sialic acid expression may reside in the specific sialic acid linkage configurations expressed in the two cell types. Terminal sialic acid residues can vary in their linkage configuration to neighboring carbohydrate residues [i.e., α(2−3), α(2−6), or α(2−8)], and importantly the different configurations have been linked to functional specificity in some systems (36). We therefore sought to determine whether the sialic acid linkages expressed by PAECs differ from that of PMVECs and probed these linkages by employing lectins specific for either an α(2−3)-linked configuration or an α(2−6)-linked configuration. PAECs and PMVECs were treated with FITC-tagged MAA (specificity: sialic acid→α2,3-Gal→β1,4-GlcNAc) to identify the presence of α(2,3)-linked sialic acids (16) or with Texas Red-tagged SNA (specificity: sialic acid→α2,6-Gal/GalNAc) to identify α(2,6)-linked sialic acids (16, 30). Both PAECs and PMVECs showed strong MAA binding (Fig. 3A), indicating the presence of α(2,3)-linked sialic acids, with strongest fluorophore staining observed in the regions of cell-cell contact. On the other hand, only PAECs exhibited strong SNA binding, reflective of α(2,6)-linked sialic acids (Fig. 3B). Although SNA staining was also observed in regions of cell-cell contact, it appeared to be somewhat more diffuse compared with the distinct MAA staining. Thus, whereas PAECs superficially express sialic acids diffusely over the cell membrane, PMVECs express sialic acids principally at the cell-cell borders. This observation was further supported by the staining with fluororescently tagged lectin from Arachis hypogaea following neuraminidase treatment. In the PAECs, the lectin bound over the entire cell membrane, whereas in PMVECs the lectin was localized to the cell-cell borders (Fig. 2B expanded regions).

From these observations, we conclude that, surficially, PAECs and PMVECs differ in their sialic acid configurations. Each cell type expresses sialic acids differently, with PAECs showing α(2,3)-linked sialic acids and PMVECs showing α(2,6)-linked sialic acids.

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Pulmonary endothelial cell barrier integrity is dependent on sialic acid presence. In the earlier set of experiments that utilized neuraminidase, we noted that, following neuraminidase treatment, there were typically fewer cells in the dish, indicating that cells had lost cell-cell and/or cell-matrix adhesions. This suggested the possibility that terminal sialic acids are important for maintenance of the endothelial barrier. Indeed, when PAECs and PMVECs were treated with neuraminidase from *Clostridium perfringens* at 1 U/ml, we observed formation of interendothelial cell gaps and loss of large areas of monolayer (Fig. 4A). When both cell types were treated over a concentration range of 0.6–1.8 U/ml neuraminidase, significant disruption of the monolayers occurred even at the lowest concentration tested (data not shown).

Neuraminidases can be obtained from biological isolates or from recombinant expression. In the present experiments, we used neuraminidase isolated from *Clostridium perfringens*. Although this preparation is highly purified, we were still concerned that other factors may be present that can also affect the endothelial barrier integrity. To address this concern, we first tested for protease activity. Using trypsin as a standard, protease activity in this preparation was well below the lowest concentration (<1 ng/ml; data not shown). Secondly, we performed a heat-inactivation study. Here we heated the neuraminidase solution at 100°C for 30 min to inactivate the enzyme, following which PAECs were treated with the inactivated enzyme at 1 U/ml for 2 h. We determined that the enzyme activity was indeed inactive because lectin from *Arachis hypogaea*, which will only bind to cells in the absence of sialic acids. In cells that were not treated with neuraminidase, the lectin did not bind; however, following neuraminidase treatment, the lectin bound to both PAECs and PMVECs, as evidenced by positive TRITC fluorescence.

**Fig. 2.** Pulmonary artery endothelial cells (PAECs) and pulmonary microvascular endothelial cells (PMVECs) express sialic acids. A: total and free sialic acids expressed by PAECs and PMVECs were quantitated. PAECs and PMVECs both contain similar amounts of free and total sialic acids. Data represent *n* = 5 for each. Values are presented as means ± SE. B: PAECs and PMVECs were treated with neuraminidase from *Clostridium perfringens* to cleave terminal sialic acids. This was followed by treatment with TRITC-tagged lectin from *Arachis hypogaea*, which will only bind to cells in the absence of sialic acids. In cells that were not treated with neuraminidase, the lectin did not bind; however, following neuraminidase treatment, the lectin bound to both PAECs and PMVECs, as evidenced by positive TRITC fluorescence. Top: transmitted light images. Bottom: TRITC fluorescence images. Areas in yellow boxes have been expanded to reveal cellular patterns of fluorescence.
treated confluent monolayers of PMVECs and PAECs with two different neuraminidases, one from *Vibrio cholerae*, which reportedly exhibits a >200:1 preference for cleavage of terminal α(2,3)-linked sialic acids (24), and one from *Clostridium perfringens*, which exhibits only a slight preference for α(2,3)-linked sialic acids over α(2,6)-linked sialic acids (3, 4). It is important to note, however, that one must be cautious in oversimplification regarding neuraminidase specificity because said specificity is dependent on both the core oligosaccharide and the protein and lipid structures that the oligosaccharides are attached to; subtle differences can dramatically influence the rate of release of different glycosidic linkages (4). Furthermore, *O*-acetylation is one of the most common modifications that occurs on sialic acids, and it has been demonstrated that monoacetlylation of the 7, 8, or 9 position of a sialic acid largely attenuates the effectiveness of neuraminidase hydrolysis; diacetlylation completely abrogates the hydrolytic ability of neuraminidases from *Clostridium perfringens* and *Vibrio cholerae* (15). Cells were treated with 1 U/ml neuraminidase for 2 and 5 h, followed by staining with fluorescently tagged lectins: FITC-tagged MAA to observe α(2,3)-linked sialic acids, and FITC-tagged SNA to observe α(2,6)-linked sialic acids. Several key observations were made. First, within 2 h of either neuraminidase treatment, the α(2,6)-linked sialic acids in PAECs were hydrolyzed as evidenced by loss of FITC-tagged SNA fluorescence (Fig. 5A). Second, we observed overall disruption of the PAEC monolayer following treatment with either neuraminidase although there were characteristic differences in what the resultant disrupted monolayer looked like. More specifically, following treatment with neuraminidase from *Clostridium perfringens*, the PAECs appeared to lose cell-cell contacts, resulting in rather evenly dispersed individual cells. On the other hand, treatment with neuraminidase from *Vibrio cholerae* resulted in large areas where there were no cells and other areas where there were still confluent cells, suggestive of loss of cell-matrix adhesions. We noted that the preparation of neuraminidase from *Vibrio cholerae*, isolated from the organism, contains trace levels of aldolase and protease activities as documented by the manufacturer. Although we know that the neuraminidase from *Vibrio cholerae* does cleave terminal sialic acids, as assessed by binding of the lectin from *Arachis hypogaea* (Fig. 5B), we do not know whether these trace level contaminants contribute to the distinctive pattern of endothelial barrier disruption. We did not use protease inhibitors in our preparations because of the concern that protease inhibitors can affect neuraminidase activity, as was previously described by Hiraiwa and colleagues (9). However, to rule out influence of other factors in this preparation, we heat-inactivated the enzyme as described above for the neuraminidase from *Clostridium perfringens*. We observed that the

Fig. 3. PAECs and PMVECs differ in their sialic acid configurations. A: confluent monolayers of PAECs and PMVECs were treated with FITC-tagged *Maackia amurensis* agglutinin (MAA). Both cell types exhibited strong, positive fluorescence indicating the presence of α(2,3)-linked sialic acids. Top: transmitted light images. Middle and bottom panels: FITC fluorescence. Bottom: expanded image of box in middle panel. B: Confluent monolayers of PAECs and PMVECs were treated with Texas Red-tagged *Sambucus nigra* agglutinin (SNA) to identify the presence of α(2,6)-linked sialic acids. PAECs exhibited strong, positive fluorescence, whereas PMVECs exhibited only a very weak fluorescence. Thus PAECs express both α(2,3)- and α(2,6)-linked sialic acids, whereas PMVECs principally express α(2,3)-linked sialic acids. Top: transmitted light images. Bottom: Texas Red fluorescence.

Fig. 4. Loss of sialic acids disrupts cell-cell and cell-matrix adhesions. A: confluent monolayers of PAECs and PMVECs were treated with neuraminidase from *Clostridium perfringens* (1 U/ml) for 2 h to cleave terminal sialic acids. Following treatment, cells were washed and imaged. Treated cells exhibited severely disrupted monolayers. Arrows point to gaps within the monolayer. B and C: PAECs were treated with heat-inactivated neuraminidase from *Clostridium perfringens* (1 U/ml) for 2 h. TRITC-tagged lectin from *Arachis hypogaea* did not bind to treated cells (B), and the monolayer was not disrupted (C).
heat-inactivated enzyme no longer cleaved sialic acids as lectin from *Arachis hypogaea* did not bind to the cells (Fig. 5C). Importantly, upon treatment of PAECs with the heat-inactivated enzyme, the endothelial barrier did not become disrupted (Fig. 5D).

At this point we knew that neuraminidase from *Vibrio cholerae* actively cleaves at least α(2,6)-linked terminal sialic acids. However, as this particular neuraminidase is reported to be more active toward α(2,3)-linked sialic acids, we were interested in determining whether these sialic acids were also cleaved. Thus we stained for α(2,3)-linked sialic acids using the FITC-tagged MAA following neuraminidase treatment. Following treatment of PAECs with neuraminidase from *Vibrio cholerae*-positive lectin, staining was still observed, indicating that not all α(2,3)-linked sialic acids were hydrolyzed (Fig. 5E). We observed the same pattern of lectin staining when the cells were treated with neuraminidase from *Clostridium perfringens*. Although at first it was...
surprising that there was still lectin binding following neuraminidase treatment, especially by the neuraminidase from *Vibrio cholerae*, this observation actually gave us insight into the diverse types of sialic acids on PAECs when we consider that certain sialic acids (e.g., O-acetylated) are highly to completely resistant to neuraminidase action (*vide supra*). Thus we conclude that PAECs express at least a subpopulation of neuraminidase-resistant (2,3)-linked sialic acids.

In PMVECs, staining for (2,6)-linked sialic acids was barely detectable in control cells and was completely absent in neuraminidase-treated cells (Fig. 6A). These results support our earlier observations that PMVECs express little surficial (2,6)-linked sialic acids. Similar to what we saw with the PAECs, in neuraminidase-treated PMVECs, staining for (2,3)-linked sialic acids was still positive, revealing that PMVECs also express a population of neuraminidase-resistant (2,3)-linked sialic acids (Fig. 6B). Because we observed positive binding of the lectin from *Arachis hypogaea* following neuraminidase treatment, and because the (2,3) linkage is the predominant one on PMVECs, it strongly suggests that indeed some (2,3)-linked sialic acids were cleaved. Furthermore, PMVEC monolayers were disrupted following treatment with either neuraminidase. Although we did not observe the complete loss of (2,3)-linked sialic acids following treatment of either neuraminidase, we did observe that, in many areas of gap formation, the (2,3)-linked sialic acid staining was nearly absent (Fig. 6C). Finally, even at 5 h postneuraminidase treatment, both PAECs and PMVECs exhibited monolayer disruption (Fig. 6D). Collectively our observations support a prominent role for terminally linked sialic acids in maintenance of endothelial barrier integrity. However, at this time we do not know whether one linkage, i.e., (2,3) or (2,6), is more important than the other in determining endothelial barrier integrity or whether further substituted (e.g., acetylated) sialic acids play a role in cell-cell and/or cell matrix adhesion.

We also performed ECIS experiments to quantitate changes in endothelial barrier integrity following neuraminidase treatment. PAECs and PMVECs were treated with three different concentrations (0.25 U/ml, 0.5 U/ml, and 1.0 U/ml) of neuraminidase from *Vibrio cholerae*, and changes in resistance were monitored over 25 h. Over this time course, we observed a dose-dependent decrease in resistance. In PAECs, at the highest dose in particular, the resistance rapidly decreased to ~60% of baseline, following which there was no further decrease in resistance (Fig. 7A). In PMVECs, at 0.5 and 1.0 U/ml neuraminidase, there was a slow, progressive decrease in resistance until it reached a plateau at 25% baseline value (Fig. 7B). We next treated PAECs and PMVECs with neuraminidase from *Clostridium perfringens*. In these experiments we used a concentration of 1 U/ml of neuraminidase from *Clostridium perfringens* (Fig. 7C). The PAEC resistance rapidly decreased to ~75% of baseline after addition of neuraminidase. This was followed by a short plateau period and then a continued slow decrease. To our surprise, the PMVEC resistance did not decrease at all following neuraminidase addition; in fact the resistance increased by ~10%. We currently have no explanation for this observation. However, although we clearly saw disruption of the monolayer in our visual microscopy experiment utilizing neuraminidase from *Clostridium perfringens*, we also noted that there were areas of intact monolayer that maintained strong cell-cell border staining of (2,3)-linked sialic acids. Additionally, it should be noted that a key difference between the microscopy experiment and the ECIS experiment is that the cells were washed four times following neuraminidase treatment for microscopy but were not washed in the ECIS experiments.

**Treatment of isolated-perfused lungs with neuraminidase from Vibrio cholerae leads to pulmonary edema.** To address whether disruption of the endothelial barrier observed in vitro also occurs in the intact pulmonary circulation, we measured the hydraulic permeability in the isolated rat lung. Lungs treated with 0.5 U/ml neuraminidase from *Vibrio cholerae* became swollen and edematous (Fig. 8A). The results shown in Fig. 8B indicate that, compared with baseline values, a 30-min neuraminidase treatment caused a severe disruption of the barrier as evidenced by an approximately eightfold increase in permeability (from 0.006 to 0.043 ml min⁻¹ cmH₂O⁻¹ × 100 g⁻¹ of predicted lung weight, respectively). A similar dramatic pattern of barrier disruption was observed after treatment with 1.0 U/ml neuraminidase (not shown). Despite the noticeable increases in permeability, neuraminidase treatment did not affect hemodynamics or airway pressures before the measurement of the final $K_f$.

To determine whether the increases in endothelial permeability in the isolated lung resulted from disruption of the micro- or macrovasculature, the lungs were fixed and processed for microscopy at the completion of the experiments. Treatment with neuraminidase from *Vibrio cholerae* caused significant fluid accumulation in the alveolar spaces, septal interstitial, and perivascular cuffs (Fig. 8C). It is important to note here that, although the formation of perivascular cuffs may be caused by protease activity, alveolar flooding is not consistent with protease activity (31). Strikingly, the high frequency of fluid accumulation in the alveolar spaces is consistent with neuraminidase activity as reported in clinical autopsy cases involving pathogenic viral infection (7, 29). The data indicate that significant and homogeneous disruption of the barrier occurred in microvascular endothelium, validating our observations from the in vitro experiments. Overall our observations reveal that terminally linked sialic acids are important for maintaining endothelial barrier integrity both in vitro and in situ.

**DISCUSSION**

The endothelial glyocalyx is comprised of a complex mixture of proteoglycans, glycoproteins, and glycolipids. Unlike polynucleotides and proteins, which are comprised of a linear chain of nucleotides and amino acids, respectively, carbohydrates form multibranched structures, further increasing potential structural complexity. To date, the structure(s) of the endothelial glyocalyx and its influence on endothelial cell function are poorly understood. Here we have begun to probe the structure-function relationship of a single terminal carbohydrate residue, sialic acid, because sialic acids are generally found at the glycan chain terminus, accessible to a singular cleavage by neuraminidases, and critically modulate the physiochemical properties of at-
Fig. 6. PMVECs express hydrolytically insensitive α(2,3)-linked sialic acids. A: PMVECs were treated for 2 h with neuraminidase from *Clostridium perfringens* (1 U/ml) or *Vibrio cholerae* (1 U/ml) followed by FITC-tagged SNA. In agreement with our earlier observations, untreated PMVECs express little to no α(2,6)-linked sialic acids. B: PMVECs treated for 2 h with neuraminidase from *Clostridium perfringens* (1 U/ml) or *Vibrio cholerae* (1 U/ml) and then treated with FITC-tagged MAA exhibited positive fluorescence, indicating that PMVECs express hydrolytically insensitive α(2,3)-linked sialic acids. This is similar to what was observed in PAECs. C: Loss of α(2,3)-linked sialic acids specifically in areas of gap formation. PAECs were treated for 2 h with neuraminidase from *Clostridium perfringens*. In areas that did not form gaps, staining for α(2,3)-linked sialic acids revealed promimentence at cell-cell borders (yellow arrows), whereas in some areas of gap formation there was loss of α(2,3)-linked sialic acids (red arrows). D: after 5-h treatment with neuraminidase from *Clostridium perfringens* or *Vibrio cholerae*, both PAECs and PMVECs exhibited disruption of the monolayer as evidenced by gap formation. For A and B, top: transmitted light images; bottom: fluorescent images.
tached glycoproteins and glycolipids. A principal observation in our study is that there is heterogeneity in sialic acid expression between endothelial cells of different vascular origins. More specifically, whereas PAECs surficially express both α(2,3)-linked and α(2,6)-linked sialic acids, PMVECs principally express α(2,3)-linked sialic acids.

A second key observation in our study is that terminally linked sialic acids are important contributors to endothelial barrier integrity. More specifically, treatment of endothelial monolayers with neuraminidases leads to endothelial barrier disruption. Indeed, our observations reveal new unknowns and thus new questions to be answered. For example, we do not yet know whether α(2,6)- or α(2,3)-linked sialic acids, or both, are critically important for barrier integrity. Additionally, it will be important to determine whether acetylated sialic acids or (2,8) dimeric-linked sialic acids play a key role in determining barrier integrity. Finally, is there a threshold of sialic acid loss that must be attained before barrier disruption occurs?

A third key observation in our study is the pattern of alveolar edema observed when isolated-perfused lungs were treated with neuraminidase from Vibrio cholerae. The diffuse alveolar damage seen in our histopathological specimens was identically observed in 25 of 34 fatalities (73.5%) reported in the 2009 influenza pandemic (7, 29). Based on these observations, it is tempting to speculate that part of the pathological mechanism of influenza infection involves neuraminidase action directly on the microvascular endothelium.

An unexplained observation in these studies is that, when PMVECs were treated with neuraminidase from Clostridium perfringens (1 U/ml), there was no decrease in resistance. To the contrary, the resistance actually increased by ~10% (Fig. 7C). We are still puzzled by this increase, especially because in our microscopy studies we did see gap formation in PMVECs following neuraminidase treatment. Perhaps this apparent discrepancy can simply be explained by the fact that, in the microscopy studies, the cells were washed four times following neuraminidase treatment, whereas they were not washed in the ECIS experiments. However, the fact that there was an actual increase in resistance suggests that there is something more going on, something we do not yet understand. Another interesting, and unexplained, observation relates to the different characteristics of barrier disruption exhibited by PAECs and PMVECs exposed to the two neuraminidases. In PAECs treated with neuraminidase from Clostridium perfringens, cells pulled apart from each other presumably through loss of cell-cell adhesions, whereas, in PMVECs treated with the same neuraminidase, the cells seemed to maintain most of the cell-cell interactions while losing cell-matrix interactions. In support of this latter observation, Kanwar and colleagues (11) demonstrated that perfusion of rat kidneys with purified neuraminidase from Clostridium perfringens led to endothelial, and epithelial, detachment from the glomerular basement membrane. The disparate responses between of PAECs and PMVECs following treatment with neuraminidase from Clostridium perfringens likely reflect the fact that multiple parameters contribute to the “specificity and kinetics of the enzyme action” (35). Key factors include the specific member of the sialic acid class involved, the anomeric configuration, its linkage to underlying sugar, and its environment (e.g., other sugars within the glycan chain and whether it is part of a bi-, tri- or higher antennary chain). Indeed, Corfield and colleagues (4) demonstrated that linkage specificity by itself is not solely sufficient to determine the rate and extent of sialic acid hydrolysis by comparison of rates of sialic acid hydrolysis using several different glycolytic substrates. Two of these substrates both exhibited an α(2,3)-linked sialic acid to an underlying galactose at the end of the oligosaccharide chain, yet neuraminidase from Vibrio cholerae ex-
hibited a more rapid hydrolysis of one substrate compared with the other. Along those same lines, compared with one substrate that possessed α(2,3)-linked sialic acids (antifreeze glycoprotein 1–5) to another substrate that possessed (2,6)-linked sialic acids (α1-acid glycoprotein), neuraminidase from Vibrio cholerae hydrolyzed the (2,6)-linked sialic acids on the α1-acid glycoprotein faster than the α(2,3)-linked sialic acids on the antifreeze glycoprotein 1–5. Thus the molecular identity and structure of the protein (or lipid) and carbohydrate chains underlying the sialic acid moieties are also important in determining the availability and rate of sialic acid hydrolysis by neuraminidase enzymes. However, we have yet to resolve the detailed underlying glycomorphology differences between the terminally linked sialic acids of PAECs and PMVECs. These questions as well as the detailed examinations of the complete glycan structures, identities, and sequences of underlying tethering proteins are the focus of our ongoing studies.

In this study, we reveal that, although both PAECs and PMVECs express sialylated oligosaccharides, the sialic acid linkages surficially expressed differ between the two cell types. Indeed, heterogeneity between PAECs and PMVECs is already well established, and our observations add yet another aspect to this heterogeneity story. In summation, our results have established that terminally linked sialic acids are critical determinants of pulmonary endothelial barrier function.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


Fig. 8. Neuraminidase from Vibrio cholerae is sufficient to cause pulmonary edema in situ. A: isolated-perfused lungs were treated with 0.5 U/ml neuraminidase from Vibrio cholerae. Photographs were taken of lung before and after treatment with neuraminidase. Following neuraminidase treatment, the lung became swollen and edematous indicative of severe disruption of the endothelial barrier. B: measurement of hydraulic permeability was measured after neuraminidase treatment revealed an 8-fold increase in permeability (from 0.006 to 0.043 ml × min⁻¹ × cmH₂O⁻¹ × 100 g⁻¹ of predicted lung weight, respectively). C: lung sections were imaged via microscopy to determine vascular segments where endothelial barrier disruption occurred. Perivascular cuffing was evident around some, but not all, larger vessels (left; blue star), whereas extensive damage was observed in the microvasculature as evidenced by fluid accumulation in the alveolar spaces and septal interstitium (right; yellow arrows).
REFERENCES


