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The endothelial glycocalyx

An important regulator of the pulmonary vascular barrier

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Abbreviations: ESL, endothelial surface layer; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; GAG, glycosaminoglycan; NO, nitric oxide; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; Kf, filtration coefficient; EBD, Evans Blue Dye; cGMP, cyclic guanosine monophosphate; V_T, tidal volume; eNOS, endothelial nitric oxide synthase

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Once thought to be a structure of small size and uncertain significance, the endothelial glycocalyx is now known to be an important regulator of endothelial function. Studies of the systemic vasculature have demonstrated that the glycocalyx forms a substantial in vivo endothelial surface layer (ESL) critical to inflammation, barrier function and mechanotransduction. The pulmonary ESL is significantly thicker than the systemic ESL, suggesting unique physiologic function. We have recently demonstrated that the pulmonary ESL regulates exposure of endothelial surface adhesion molecules, thereby serving as a barrier to neutrophil adhesion and extravasation. While the pulmonary ESL is not a critical structural component of the endothelial barrier to fluid and protein, it serves a major role in the mechanotransduction of vascular pressure, with impact on the active regulation of endothelial permeability. It is likely that the ESL serves numerous additional functions in vascular physiology, representing a fertile area for future investigation.

The maintenance of a selective endothelial barrier regulating fluid, protein and cellular extravasation is essential to normal tissue function. Endothelial barrier function is particularly critical within the pulmonary circulation, where interstitial edema can have profound impact on gas diffusion across the alveolar septum, leading to hypoxemia and multiple systemic consequences. Acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) are critical illnesses emblematic of such untoward effects of pulmonary endothelial barrier dysfunction. In ALI/ARDS, inflammatory stimuli lead to increased endothelial and epithelial barrier permeability, with consequent neutrophilic pulmonary edema, severe hypoxemia and significant morbidity and mortality.[1] Despite four decades of intense investigation, however, there remains no clinically-efficacious, pathophysiology-targeted treatment for ALI/ARDS, reflecting an incomplete understanding of the mechanisms underlying pulmonary endothelial barrier dysfunction during lung injury.

ALI/ARDS investigations to date have often focused on intra-endothelial signaling cascades underlying paracellular and transcellular transit, such as regulation of tight and adherens junctional integrity as well as endothelial cytoskeletal contraction.[2,3] However, endothelial barrier integrity is also determined by extracellular structures, including the vascular basement membrane as well as the endothelial glycocalyx. The glycocalyx is a complex layer of sialic acid-containing glycoproteins, membrane-bound proteoglycans (e.g., syndecans, glypicans) and associated glycosaminoglycans (GAGs, including heparan sulfate and hyaluronic acid) lining the intimal surface of blood vessels (**Fig. 1A**).[3,4] The relevance of the glycocalyx to barrier function has been generally underappreciated, in part due to a long-standing perception that the glycocalyx was a small, inconsequential structure.[3] With the development of in vivo (intravital) microscopy techniques, it became apparent that this perception was

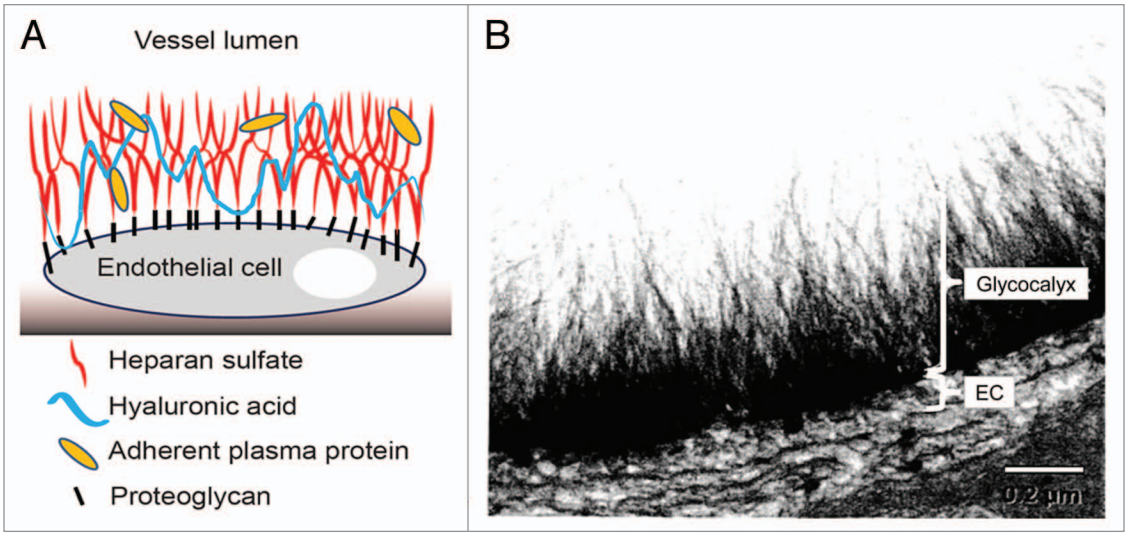

Figure 1. Structure of the endothelial surface layer. (**A**) The endothelial surface layer (ESL, the in vivo manifestation of the endothelial glycocalyx) is comprised of a layer of proteoglycans and associated glycosaminoglycans (heparan sulfate, hyaluronic acid) lining the intimal surface. Sialic acid-containing glycoproteins (not pictured) and adherent plasma proteins additionally contribute to glycocalyx/ESL structure. Image not to scale. Adapted from Schmidt EP et al. Nat Med 2012; 18:1217–23. (**B**) Glycocalyx/ESL thickness is substantially greater than endothelial cell (EC) thickness, as demonstrated in a transmission electron micrograph of a goat coronary capillary (prepared using glycocalyx-sparing fixation techniques). Scale bar: 0.2 μm. Image reproduced with permission from van den Berg BM et al. Pharmacological Reports 2006; 58:suppl. 75–80, copyright 2006 Polish Academy of Sciences.

inaccurate: in vivo, the glycocalyx forms a substantial (0.5–1 μm in systemic vessels) endothelial surface layer (ESL) not seen in most in vitro preparations.[5,6] Interestingly, this thickness dwarfs that of the endothelial cell itself (**Fig. 1B**).[7,8] For purposes of this discussion, we will define the "ESL" as the in vivo manifestation of the endothelial glycocalyx, reserving the term "glycocalyx" to refer to the membrane-bound proteoglycans, GAGs and glycoproteins comprising the structural framework underlying ESL integrity.[7]

With the recognition of its substantial size, increasing scientific attention has been devoted to determining the physiologic importance of the ESL (and, accordingly, the underlying glycocalyx structures critical to ESL integrity). In studies primarily performed using systemic microvessels (e.g., the mesenteric and/or cremasteric microcirculations), the ESL was found to be a key regulator of multiple facets of endothelial function. ESL integrity controls neutrophil extravasation via multiple mechanisms, including the regulation of endothelial adhesion marker exposure[9] as well as the presentation of chemokines that direct neutrophil intraluminal crawling.[10] Highly-sulfated glycocalyx GAGs contribute to a negatively-charged ESL, creating a transendothelial protein gradient critical to the Starling forces that define fluid transit.[11] This charge barrier is particularly important to the fenestrated endothelium of the renal glomerulus, with ESL degradation potentially contributing to diabetic nephropathy and other disorders of glomerular function.[12] Additionally, glycocalyx GAGs are essential to the mechanotransduction of shear stress into nitric oxide (NO)-mediated vasorelaxation.[13]

These functions of the glycocalyx/ESL, determined primarily using models of the systemic circulation, have potential relevance to the pulmonary vascular dysfunction characteristic of ALI/ARDS. Indeed, the pulmonary vascular pathophysiology of ALI/ARDS is characterized by augmented neutrophil adhesion and transmigration,[1] increased endothelial permeability to protein and fluid,[1] and aberrant vasorelaxation[14] and NO signaling.[15] It may be incorrect, however, to assume that ESL functions observed within the systemic vasculature similarly exist within the pulmonary circulation. Indeed, the pulmonary ESL markedly differs from the ESL of systemic vessels. Using intravital microscopy of wild-type C57BL/6 mice,[16] we noted a mean pulmonary microvascular ESL thickness of 1.67 μm, dramatically greater than cremasteric ESL thickness observed by us[16] (0.67 μm) and others.[17] Even the in vitro glycocalyx, thought to be miniscule in cultured systemic endothelial cell preparations,[5,6] is substantial (2.8 μm thickness) on bovine lung microvascular endothelial cell monolayers.[18] The physiologic significance of differences in systemic and pulmonary ESL thickness is largely unexplored. Given the ability of oxygen to diffuse distances ranging from 30 μm (brain) to 300 μm (muscle), it appears unlikely that the 1 μm-greater thickness of the pulmonary ESL significantly impacts erythrocyte oxygenation, particularly given the high alveolar partial pressure of oxygen driving diffusion into pulmonary capillaries.[19] However, this difference in ESL thickness (or, more precisely, the glycocalyx structures underlying this difference) may be sufficient to alternatively impact other endothelial barrier functions.

The remainder of this article will highlight the evolving understanding of the ESL as a regulator of the pulmonary vascular barrier, reviewing recently-published investigations as well as presenting new data derived from ex vivo and in vivo mouse models.

The Pulmonary Glycocalyx as a Barrier to Neutrophil Extravasation

The ESL is ideally positioned to serve as the interface between circulating inflammatory cells and the endothelial surface. Inflammatory diseases such as septic shock (a major cause of ALI/ARDS) are characterized by increased plasma concentrations of GAG fragments, suggesting that glycocalyx degradation is associated with inflammatory tissue injury.[20,21] Given the known association of (experimental) glycocalyx degradation with neutrophil adhesion in systemic microvessels,[3] we hypothesized that sepsis-induced pulmonary ESL loss would mediate the onset of pulmonary neutrophil adhesion and (consequently) inflammatory lung injury. Indeed, we found that in experimental sepsis, pulmonary ESL loss was rapid and dramatic, with a significant decrease in thickness (1.67 to less than 0.5 μm) occurring within 30 min of the onset of endotoxemia.[16] This degradation was associated with post-translational activation of constitutively-expressed endothelial heparanase, a glucuronidase specific for heparan sulfate, the predominant glycocalyx GAG. Heparanase-mediated ESL loss (further amplified by a later increase in total heparanase expression) served to expose pulmonary endothelial surface adhesion molecules (e.g., ICAM-1, VCAM-1) to circulating activated neutrophils, facilitating adherence and extravasation in response to an inciting inflammatory stimulus (**Fig. 2**). Heparanase inhibition, in turn, prevented adhesion molecule exposure and attenuated inflammatory injury. These findings were supported by human data demonstrating increased pulmonary heparanase content in lung biopsies with diffuse alveolar damage (the histologic manifestation of ALI/ARDS) and increased heparan sulfate degradation activity in plasma collected from patients with sepsis.[16] Taken together, our findings suggested that a fundamental role of the pulmonary ESL is to regulate endothelial surface exposure and thus control neutrophil influx into the lung.[16]

While the concept of ESL regulation of endothelial surface exposure is attractive in its simplicity, there likely exist multiple additional mechanisms by which the pulmonary ESL influences neutrophil adhesion and transmigration. In systemic vessels, endothelial heparan sulfates enable neutrophil slow rolling by serving as a ligand for leukocyte L-selectin, a function dependent upon GAG sulfation.[22] The relevance of this finding to pulmonary inflammation is uncertain: while L-selectin has been implicated in lung injury pathogenesis[23] and pulmonary microvascular neutrophil margination,[24] neither L-selectin nor leukocyte rolling is critical to neutrophil extravasation from the pulmonary microcirculation.[24,25] Alternatively, pulmonary glycocalyx GAGs may function as repositories for neutrophil-stimulating chemokines during inflammatory lung injury.[22,26] Heparan sulfate may also enable the trafficking of chemokines from the basolateral to luminal surface of mouse lung endothelial cells.[22] Indeed, the ability of vessels to adapt glycocalyx structures during inflammation suggests a complex contribution of the ESL to the regulation of neutrophil extravasation.[27]

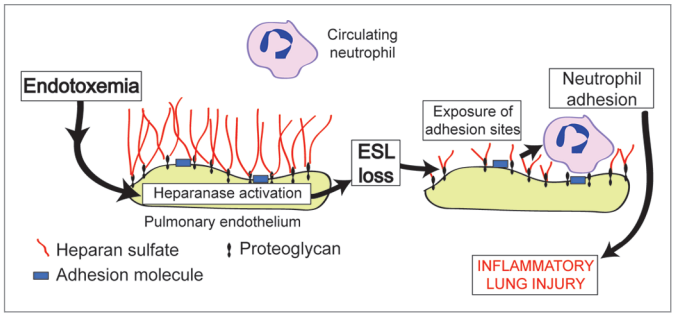

Figure 2. Endothelial surface layer regulation of adhesion molecule exposure during sepsis. During health, the endothelial surface layer (ESL) serves as a barrier separating the pulmonary endothelial surface from circulating neutrophils. During endotoxemia, activated heparanase cleaves the ESL, exposing pulmonary endothelial surface adhesion molecules, leading to neutrophil adhesion and inflammatory lung injury. Adapted from Schmidt EP et al. Nat Med 2012 18:1217–23.

The Pulmonary Glycocalyx as a Barrier to Fluid and Protein Extravasation

In systemic vessels, the ESL serves as an important structural component of the endothelial barrier opposing fluid and protein extravasation. It has been proposed that glycocalyx GAGs, by forming a charged meshwork overlying cell-cell junctions, determine the transvascular oncotic pressure gradient which contributes to the Starling regulation of fluid flux.[11] This "modified Starling" theory (in which the ESL, not cell-cell junctions, dictates the transvascular oncotic gradient) has been supported by multiple studies of the systemic circulation, in which glycocalyx degradation led to increased protein and fluid permeability.[28-30]

It is uncertain if the ESL similarly serves as a structural component of the pulmonary endothelial barrier to fluid and protein. While an in vitro study of bovine lung microvascular endothelial cells suggested that glycocalyx heparan sulfate content contributed to the baseline endothelial barrier to fluid,[31] this finding was not replicated in an ex vivo isolated rat lung preparation.[32] We similarly determined that in isolated mouse lungs perfused with 4% Evans Blue Dye (EBD)-labeled albumin, neither fluid (filtration coefficient, Kf) nor protein (lung EBD-albumin extravasation) permeability was altered by glycocalyx degradation (**Fig. 3A and B**). Furthermore, degradation of glycocalyx heparan sulfates in vivo (using intravenous heparinase-III, a heparan sulfate-specific bacterial glucuronidase that rapidly degrades the pulmonary ESL[16]) did not increase lung edema in mice (**Fig. 3C**). These findings suggest that, in contrast to systemic vessels, pulmonary glycocalyx GAGs do not structurally (i.e., passively) contribute to the baseline in vivo pulmonary endothelial barrier to fluid and protein. The mechanisms underlying this counterintuitive finding (indeed, the larger pulmonary ESL would be expected to serve as a more robust passive barrier) are unknown.

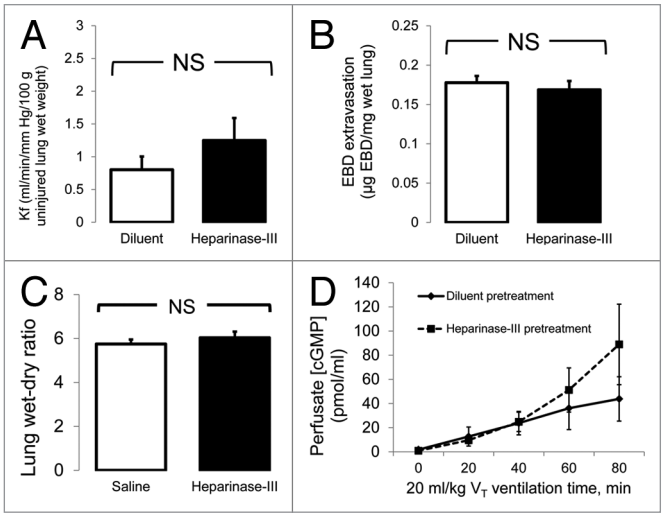

Figure 3. Glycocalyx impact on pulmonary endothelial function. (**A and B**) Isolated C57BL/6 mouse lungs (n = 4–5 per group) were perfused for 30 min with diluent (4% BSA in BMOC-3, Invitrogen) or heparinase-III (50 mU/ml, Sigma) at an isogravimetric state, avoiding enzyme extravasation. Heparinase-III decreased vascular heparan sulfate content by 40% in lung sections (data not shown), similar to loss in septic ALI.[16] Perfusate was then changed to BMOC-3 with 4% Evans Blue Dye (EBD)-labeled albumin. Heparan sulfate degradation did not alter endothelial permeability to fluid [filtration coefficient, Kf, (**A**)] or protein [EBD extravasation during the 30 min (two 15 min pressure steps) Kf measurement, (**B**)]. Isolated lung preparation and measurements performed as previously described.[15] (**C**) C57BL/6 mice (n = 5–9 per group) were treated with intravenous saline (200 μl) or heparinase-III (1 unit in 200 μl, sufficient to induce ESL degradation[16]). Two hours later, lungs were harvested for wet-dry ratio measurement,[15] an index of lung edema. (**D**) Isolated mouse lungs were perfused with diluent or heparinase-III, as described in (a,b). Perfusate was then changed to BMOC-3 media with 100 μM isobutyl methylxanthine (preventing cGMP degradation[15]), and lungs were ventilated with 20 ml/kg tidal volumes (V_T). Perfusate cGMP measured as previously described.[15]

Of note, a recent study has addressed the contribution of non-GAG components of the endothelial glycocalyx to vascular barrier function. Degradation of sialic acids from endothelial surface glycoproteins led to increased endothelial permeability in vitro and ex vivo.[33] It is unclear, however, if this effect on permeability reflects a passive (i.e., structural) contribution of sialic acids to the vascular barrier or if loss of sialic acid residues triggers endothelial signaling cascades that lead to hyperpermeability. Interestingly, cell surface sialic acid degradation was associated with endothelial cell detachment from the basement membrane, suggesting transduction of an apical signal to the basolateral surface.[33] Furthermore, use of certain neuraminidases to degrade sialic acids was paradoxically associated with a strengthening of barrier function.[33] These findings suggest a dynamic role of sialic acid-containing glycoproteins in the active regulation of barrier function and not simply a passive structural contribution.

The Pulmonary Glycocalyx and Nitric Oxide-Induced Endothelial Permeability

While pulmonary glycocalyx GAGs may not structurally contribute to the baseline endothelial barrier to fluid and protein, their loss could initiate signaling cascades that ultimately alter vascular permeability. GAGs such as heparan sulfate and hyaluronic acid are vital to the transduction of vascular shear stress into the NO-mediated vasorelaxation of systemic vessels.[13,34,35] The mechanisms underlying glycocalyx control of NO-mediated vasorelaxation are uncertain, but may reflect an oxidant-mediated control of NO bioavailability[36] or the potential ability of transmembrane syndecans (via intracellular kinase domains[37]) to regulate eNOS activity. As NO is known to contribute to the control of pulmonary endothelial permeability,[15] the mechanotransductive properties of the ESL may have relevance to the pulmonary barrier dysfunction characteristic of ALI/ARDS.

The pulmonary glycocalyx/ESL has several NO-dependent mechanotransductive capabilities. Bovine lung microvascular endothelial cells accommodate a trans-monolayer pressure stimulus by increasing permeability in a NO-dependent fashion.[31] This transendothelial pressure gradient is sensed (i.e., "transduced") by cell-surface heparan sulfates, as the pressure-induced increase in permeability was lost after monolayer treatment with heparinase-III. These in vitro findings were corroborated using an isolated, perfused rat lung model, in which increased vascular pressure (i.e., hoop stretch) augmented endothelial permeability in a heparan sulfate-dependent manner.[32] These findings are ostensibly relevant to lung injury, given the potential importance of vascular distension in determining ALI/ARDS outcomes.[38]

Of note, other physical forces besides vascular distension (e.g., tidal volume[39]) are highly relevant to ALI/ARDS outcomes. Indeed, high tidal volume ventilation induces endothelial NO production which, in turn, increases endothelial cyclic guanosine monophosphate (cGMP) concentrations.[15] Given that cGMP production may contribute to endothelial dysfunction,[15] the ability of the ESL to transduce ventilatory stretch into NO production could have importance in ALI/ARDS pathogenesis. Interestingly, we found that glycocalyx degradation did not prevent stretch-induced cGMP production, suggesting that the ESL is not necessary for the NO-mediated mechanotransduction of tidal volume (**Fig. 3D**). It is uncertain whether ESL loss can contribute to the pathogenesis of ventilator-induced lung injury via alternative mechanisms.

Unexplored Impact of the ESL on the Pulmonary Endothelial Barrier

Appreciation for the biologic significance of the ESL has grown dramatically in the last decade. What was once thought to be a trifling structure is now known to impact multiple facets of tissue function, including inflammatory cell adhesion, endothelial permeability and NO signaling. The importance of the ESL likely extends well beyond these functions.

Indeed, the glycocalyx holds staggering structural complexity: the sulfation pattern of GAG chains is sufficiently complex that potentially no two chains are identical.[40] This diversity, which exceeds that of nucleic acids,[26] may similarly encode biologic information. Tissue-specific heparan sulfation patterns may serve as a GAG "fingerprint" individualized to the specific biologic needs of a vascular bed.[41] The importance of this fingerprint has not yet been appreciated, representing an exciting opportunity for future high-impact investigations of vascular physiology.

Finally, it is important to emphasize the unexplored therapeutic potential of ESL biology in diseases such as ALI/ARDS. As discussed above, pulmonary ESL functions such as neutrophil adhesion, endothelial transduction of physical stress and NO signaling are important known contributors to ALI/ARDS pathogenesis. As circulating glycocalyx degradation products can be readily detected in human patients,[20,21] ESL loss may serve as a viable biomarker predicting impending organ injury during systemic inflammatory states such as sepsis. Once detected, ESL loss may be attenuated using agents (e.g., heparin[16]) well-tolerated even in critically ill patients. Indeed, heparin (administered at clinically-therapeutic doses) has been suggested to improve patient outcomes in septic shock.[42] Future translational investigations of ESL biology, therefore, may not only yield significant advances in the understanding of vascular physiology, but may also have major impact upon the care of the critically ill.

Disclosure of Potential Conflicts of Interest

The authors have no conflict of interest with organizations with financial interest in the subject matter.

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