

Review Article

Hyaluronan regulation of vascular integrity

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Abstract: Vascular integrity or the maintenance of blood vessel continuity is a fundamental process regulated, in part, by the endothelial glycocalyx and cell-cell junctions. Defects in endothelial barrier function are an initiating factor in several disease processes including atherosclerosis, ischemia/reperfusion, tumor angiogenesis, cancer metastasis, diabetes, sepsis and acute lung injury. The glycosaminoglycan, hyaluronan (HA), maintains vascular integrity through endothelial glycocalyx modulation, caveolin-enriched microdomain regulation and interaction with endothelial HA binding proteins. Certain disease states increase hyaluronidase activity and reactive oxygen species (ROS) generation which break down high molecular weight HA to low molecular weight fragments causing damage to the endothelial glycocalyx. Further, these HA fragments can activate specific HA binding proteins upregulated in vascular disease to promote actin cytoskeletal reorganization and inhibition of endothelial cell-cell contacts. This review focuses on the crucial role of HA in vascular integrity and how HA degradation promotes vascular barrier disruption.

Keywords: Endothelial permeability, glycocalyx, caveolin-enriched microdomain, actin cytoskeleton, CD44, HBP2, versican, TLR2, TLR4, caveolin-1

Introduction

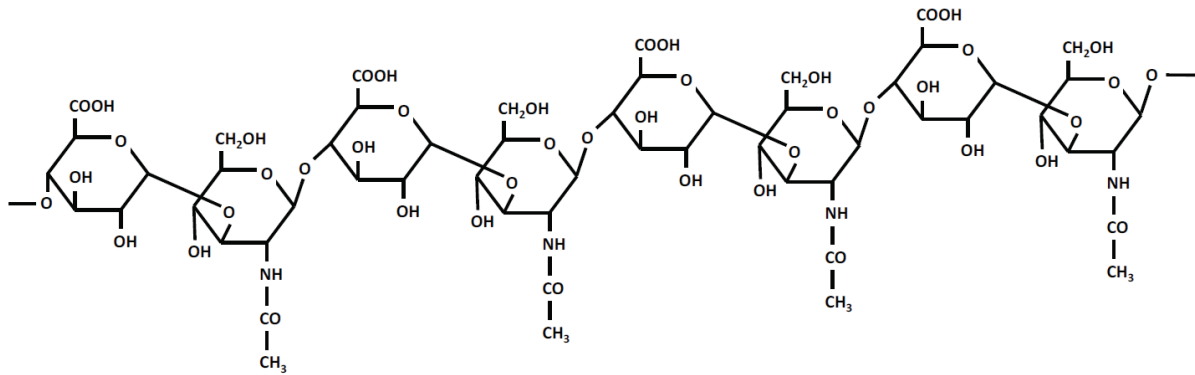
Vascular integrity (i.e. the maintenance of blood vessel continuity) is required for normal cardiovascular homeostasis [1, 2]. Several mechanisms regulate basal vascular integrity including the endothelial glycocalyx, a meshwork of hyaluronan (HA), proteoglycans, glycolipids and proteins between the vascular luminal space and the endothelial cell (EC) surface, endothelial cell-cell junctions which are controlled by tight junctions, adherens junctions and caveolin-enriched microdomains (CEM) [1-9]. Certain pathologies induce degradation of the glycocalyx and disruption of EC-EC junctions causing leakage of fluids and proteins into the underlying tissue [1, 2, 10-15].

The major non-sulfated glycosaminoglycan in most tissues, hyaluronan (HA), plays a fundamental role in the maintenance of vascular integrity [4, 16-28]. HA is composed of a linear repeat of disaccharide units consisting of D-glucuronic acid and N-acetylglucosamine [19-22] (**Figure 1**). The major form of HA *in vivo*, high molecular weight HA (HMW-HA), has a mo-

lecular weight >1 million Da. HMW-HA exhibits a random coil structure that can expand in aqueous solutions [23-25]. Aqueous HMW-HA is highly viscous and elastic, properties which contribute to its filtering functions in the glycocalyx [26-28]. HA is a dynamic molecule with a high rate of metabolism. In humans, the turnover rate for HA is 5 grams per day of the 15 total grams in the body [29]. The majority of HA in the vasculature is incorporated into the endothelial glycocalyx and the extracellular matrix of the underlying tissue [17, 27, 28, 30]. The levels of soluble HA are low in normal human plasma due to rapid removal by the liver and kidneys [29].

In EC, as in other cell types, HA is synthesized by hyaluronan synthases (HAS) [31]. The three main HAS (HAS1, HAS2 and HAS3) differ in the Km values for their substrates (D-glucuronic acid and N-acetylglucosamine) leading to differential rates of hyaluronan synthesis and secretion from the plasma membrane [32]. HAS1 and HAS2 produce HA with a molecular weight > 500 kDa and HAS3 produces < 500 kDa HA [31]. HAS2 deletion results in embryonic lethal-

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((1-4)-beta-N-acetyl-D-glucosamino)-beta-(1-3)-D-glucuronan

Figure 1. The Chemical Structure of hyaluronan (HA). HA is composed of linear repeating disaccharide units consisting of D-glucuronic acid and N-acetylglucosamine [19].

ity due to cardiac developmental defects and vascular abnormalities, effects which are rescued by addition of exogenous HMW-HA [33,34].

HA is degraded in certain disease states by hyaluronidases and ROS to produce lower molecular weight fragments (<500 kDa) (24). There are six hyaluronidase genes encoding HYAL-1,2,3,4, PHYAL1 (a pseudogene) and PH-20 [35,36]. HYAL enzymes have different cellular localization and optimal pH activity which can lead to generation of different sized HA fragments [35, 37-39]. Degradation of HA in the vasculature occurs in multiple pathological conditions [24, 39, 40]. Our laboratory has demonstrated that HMW-HA (~1 million Da) promoted Rac1-dependent cortical actin formation and EC barrier enhancement while low molecular weight HA (LMW-HA, ~2,500 Da) induced RhoA-dependent actin stress fiber formation and disruption of the EC barrier *in vitro* [18] (**Figure 2**). The differential mechanisms of HA's regulation of vascular integrity in normal and disease states are discussed below.

HA regulation of the endothelial glycocalyx

The endothelial glycocalyx is a negatively charged "mesh" of membrane glycoproteins, proteoglycans and glycosaminoglycans (including HA) which is located on the luminal side of the endothelium in all blood vessels [8, 13, 15, 27, 41]. Endothelial glycocalyx thickness varies with vessel size and can range from 0.5 μm in capillaries up to 4.5 μm in the carotid arteries [8]. Newly synthesized HA may be incor-

porated in to the glycocalyx as it is extruded from the cell membrane and then bound by CD44 or other HA-binding proteins. There are several novel techniques currently utilized to determine the contribution of HA to the EC glycocalyx including fluorescent correlation spectroscopy and atomic force microscopy [14, 41-44]. The endothelial glycocalyx also incorporates serum proteins such as albumin, fibrinogen and extracellular superoxide dismutase [15]. The glycocalyx has a number of important vasculoprotective functions *in vivo*, including a) regulation of vascular permeability, b) modulation of leukocyte rolling and adhesion, c) transduction of shear stress leading to NO release and d) inhibition of coagulation [13, 14, 45-48].

Vascular permeability

The glycocalyx can be described as a molecular sieve along the capillary wall, with pore size dependent on the spacing between the glycocalyx fibers. Proteins, peptides and even lipids may penetrate this "sieve" to various degrees thereby establishing a dynamic equilibrium between components in the flowing blood and those retained within the glycocalyx. According to this model proposed by Adamson et al., an almost protein-free space should exist beneath the outer face of the glycocalyx next to the luminal surface of the endothelial cell as plasma is forced outwards hydrostatically, but proteins are retained or excluded from the glycocalyx [49]. Because the fluid passing through the glycocalyx is therefore extremely low in protein, an inwardly directed oncotic gradient will be generated across the glycocalyx limiting net out-

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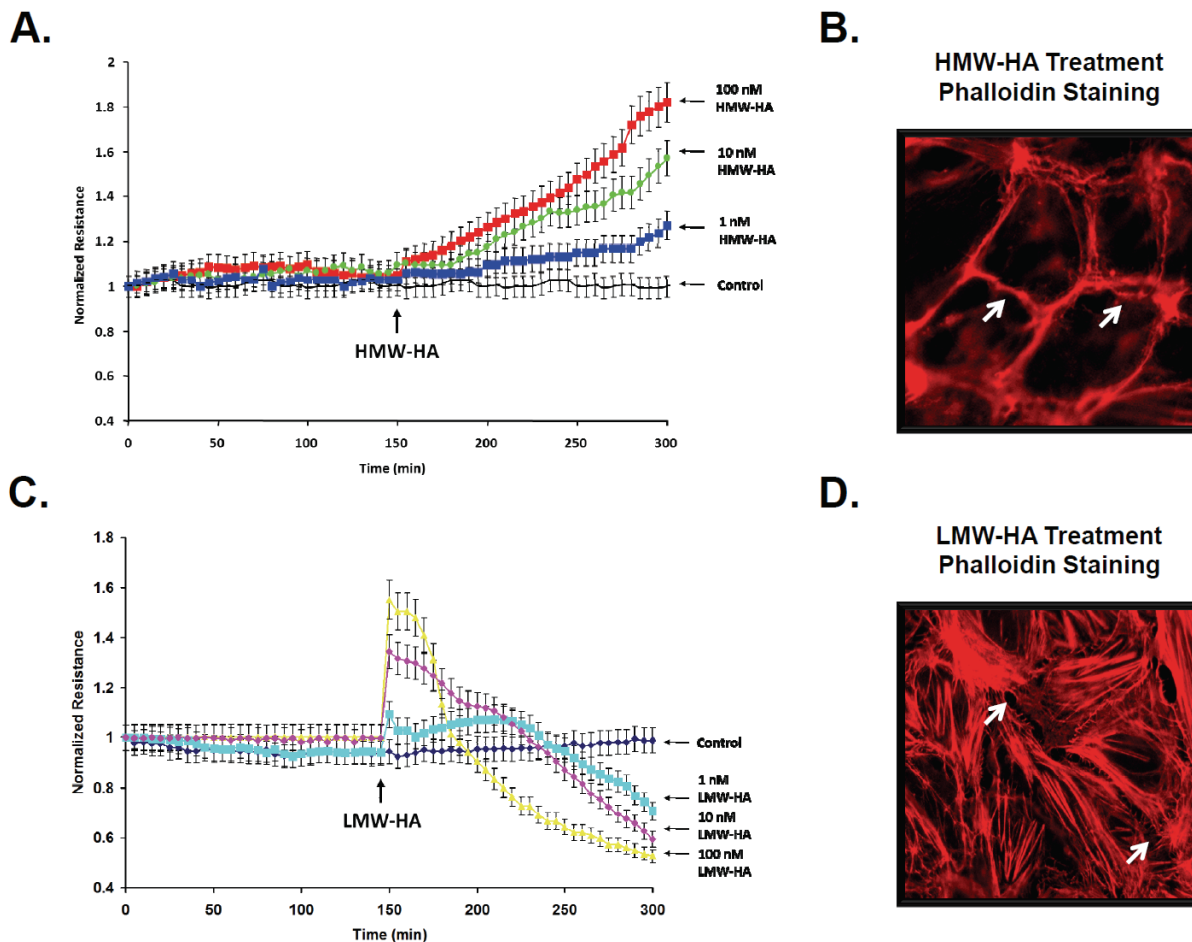


Figure 2. Hyaluronan Regulation of Endothelial Barrier Function and the Actin Cytoskeleton. HMW-HA (~1 million Da) induces a dose-dependent increase in human pulmonary microvascular EC barrier function (A) and promotes cortical actin ring formation (B). The arrows indicate areas of cortical actin associated with EC contacts. In contrast, LMW-HA (~2,500 Da) promotes a biphasic response resulting in EC barrier disruption (C) and actin stress fiber formation (D). The arrows indicate gap formations between ECs. This research was originally published in *The Journal of Biological Chemistry* (Singleton et al., *J. Biol. Chem.*, 2006, 10;281(45):34381-93) © the American Society for Biochemistry and Molecular Biology [18].

flow of filtrate towards the interstitial space [15, 50-52]. Degradation of the coronary glycocalyx with hyaluronidase leads to myocardial edema in perfused rat hearts [53]. Gao and Lipowsky also investigated the effects of hyaluronidase treatment on glycocalyx permeability in post-capillary venules in the rat [27]. They reported that although heparinase, chondroitinase and hyaluronidase treatment all decreased the thickness of the glycocalyx, only treatment with hyaluronidase and chondroitinase increased the diffusion of FITC to the sublayer of the glycocalyx [27]. This indicates that HA and chondroitin (CN) contribute a significantly greater amount to glycocalyx permeability than HS, and

may indicate HS is more concentrated in the upper portion of the glycocalyx while HA and CN contribute to a denser sublayer adjacent to the EC.

Leukocyte rolling and adhesion

The dimensions of the glycocalyx are such that it can sterically hinder firm attachment of leukocytes and platelets to the endothelial cells [52, 54-56]. Disruption or shedding of the glycocalyx leads to increased leukocyte adhesion [56, 57]. Inhibition of hyaluronan synthesis using 4-methylumbelliferone (4-MU) in the ApoE deficient mouse led to decreased glycocalyx forma-

tion and increased adhesion of leukocytes in the carotid artery which ultimately led to increased atherosclerosis [58]. HA fragments, which may be released with glycocalyx disruption, act as pro-inflammatory molecules [59].

Shear stress

HA is an important factor in mechanosensing and mediating nitric oxide (NO) release within the endothelium [60-62]. Mochizuki et al., compared NO levels in isolated canine femoral arteries before and after hyaluronidase perfusion to degrade the HA component of the glycocalyx [60]. The NO production rate increased linearly with perfusion rate before enzyme treatment; and they observed a significant decrease in the rate of NO production following hyaluronidase treatment (0.084 to 0.009 nmol/ml) [60]. However acetylcholine-induced NO production was unaffected [63]. A later study by Pahakis et al., reported that removal of hyaluronan, heparan sulfate or sialic acid but not chondroitin sulfate could individually block shear induced NO production in primary bovine endothelial cells [61]. However *in vitro* studies using human vascular umbilical endothelial cells demonstrated that shear stress leads to an increase in hyaluronan (but not heparan sulfate) incorporation into the glycocalyx and the media of cultured cells [30]. Taken together, these studies would seem to indicate that hyaluronan is a key component in transducing shear stress leading to NO production in the endothelium.

Inhibition of coagulation

Closely linked to its role in leukocyte adhesion and shear stress mechanosensing is the role of the glycocalyx in inhibiting coagulation. The glycocalyx harbors a wide range of proteins involved in coagulation, fibrinolysis and haemostasis including antithrombin III, thrombomodulin and tissue factor pathway inhibitor which all help to maintain an anti-thrombotic environment [8]. However, damage to the glycocalyx results in a loss of these factors (and also exposes the endothelium to platelet adhesion) and is believed to be a first step in the development of a pro-thrombotic environment [8].

Glycocalyx recovery after injury

Although there is currently no data available on rates of glycocalyx recovery in humans, a study

using mouse models following hyaluronidase or TNF- α treatment indicates that it can take up to seven days for full reconstitution of the glycocalyx to occur [42]. Mulivor and Lipowsky have demonstrated that an infusion of pertussis toxin, which inhibits G-protein stimulated shedding of glycosaminoglycan chains could significantly attenuate glycocalyx loss in response to either ischemia/reperfusion or N-formylmethionyl-leucyl-phenylalanine (fMLP) administration [12]. A number of studies have used a perfusion of exogenous HMW-HA to restore the glycocalyx following degradation with hyaluronidase [28] or in response to ischemia/reperfusion injury [64]. In contrast, HA degradation products can induce ROS production (and vice versa), a crucial factor in glycocalyx degradation in numerous vascular disease processes [40, 64, 65]. Perhaps most promising from a clinical standpoint, Nieuwdrop et al., have used an infusion of the antioxidant NAC to protect against hyperglycemic induced glycocalyx shedding, which further indicates a role for ROS in glycocalyx disruption [14].

HA regulation of endothelial caveolin-enriched microdomain dynamics

In endothelial cells, as in many other cell types, there are specialized cholesterol- and sphingolipid/glycolipid-enriched microdomains called lipid rafts which have been implicated in numerous cellular functions [66-69]. EC contain a subset of lipid rafts termed caveolin-enriched microdomains (CEM) which are 50 to 100 nm plasma membrane microdomains containing the scaffolding protein, caveolin-1 [6, 18, 69, 70]. We and others have demonstrated that CEM are important regulators of vascular integrity. Caveolin-1 knockout mice do not have CEM (caveolae) formation in EC and exhibit microvascular hyper-permeability [71-74].

CEM are crucial for HA regulation of vascular integrity [4, 18]. Our previous published data indicate that HMW-HA recruited CEM containing the HA binding protein, CD44, and actin cytoskeletal regulatory proteins to areas of EC-EC contact [4, 18] (**Figure 3**). Abolishing CEM formation by cholesterol depletion (M β CD) or silencing caveolin-1 expression blocked HMW-HA-mediated human pulmonary microvascular EC Rac1 activation, cortical actin formation and barrier enhancement *in vitro* [18]. In addition, we observed that HMW-HA protection from LPS-

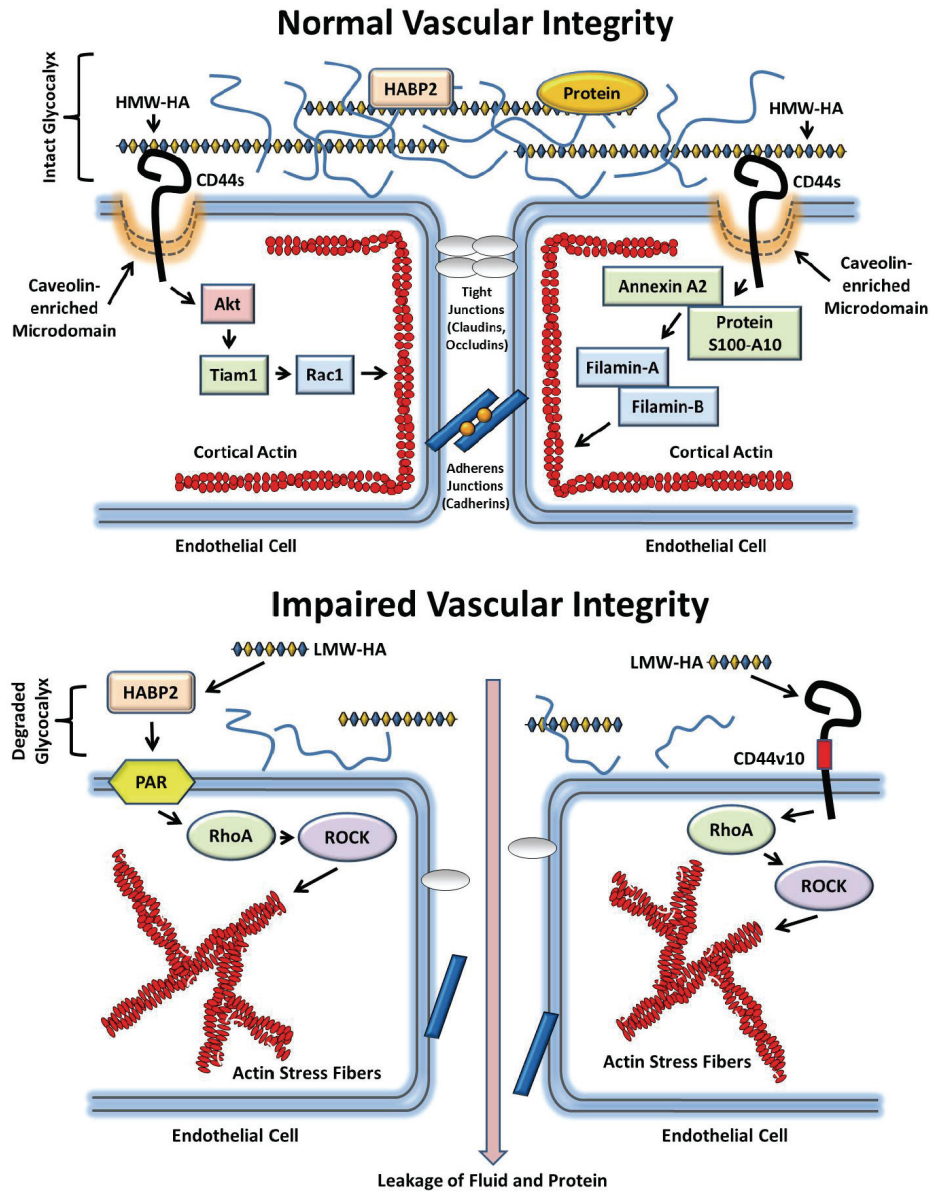


Figure 3. Hyaluronan Regulation of Normal and Impaired Vascular Integrity. High molecular weight hyaluronan (HMW-HA), the major non-sulfated glycosaminoglycan in the body, maintains vascular integrity through endothelial glycoalyx modulation, caveolin-enriched microdomain (CEM) regulation and interaction with endothelial HA binding proteins (upper panel). In the glycoalyx, HMW-HA interacts with proteoglycans (including versican) and glycoproteins to form a negatively charged “mesh” located on the luminal side of the endothelium in all blood vessels [8]. This glycoalyx regulates vascular permeability and incorporates serum proteins such as albumin, fibrinogen and extracellular superoxide dismutase [15]. HMW-HA binds to and inhibits the EC barrier disrupting activity of the extracellular serine protease HABP2 [130]. In addition, HMW-HA binds to the transmembrane receptor, CD44s (standard form), in CEM which results in Akt-mediated Tiam1 activation and Rac1-GTP formation leading to cortical actin formation and strengthening of EC-EC contacts [18]. Further, HMW-HA recruits several other actin regulatory proteins to CEM including annexin A2, protein S100-A10, filamin-A and filamin-B which enhance cortical actin formation and vascular integrity [4]. In disease states such as atherosclerosis, ischemia/reperfusion, tumor angiogenesis, cancer metastasis, diabetes, sepsis and acute lung injury, there is impaired vascular integrity (lower panel). Damage to the endothelium generates reactive oxygen species (ROS) and hyaluronidase activation lead to generation of low molecular weight HA fragments (LMW-HA) [17, 28, 100]. In addition to CD44v10 (variant 10) ligation, LMW-HA binds to and activates HABP2 which induces protease-activated receptor (PAR) activation in EC [18, 130]. These events promote RhoA-GTP formation and stimulation of rho kinase (ROCK) activity leading to actin stress fiber formation and EC barrier disruption [18, 130]. Disruption of the endothelium promotes leakage of fluid and protein into the underlying tissue [1, 2, 6, 106, 134].

induced pulmonary vascular hyper-permeability was blocked in the caveolin-1 knockout mouse [4]. These data indicate the crucial regulatory role of caveolin-1 and CEM in HMW-HA-mediated enhancement of vascular integrity.

HA involvement in vascular disease

In certain vascular disease states, HMW-HA is degraded by hyaluronidases and ROS to low molecular weight fragments [20]. The differential activities of HMW-HA and its degradation products on vascular integrity are due to changes in endothelial glycocalyx dynamics, regulation of EC-EC contacts and CEM dynamics and the relative expression of specific HA-binding proteins in normal and disease states which are discussed below.

Atherosclerosis

The initiating step in atherosclerosis is EC barrier dysfunction followed by accumulation of low density lipoproteins, cholesterol and monocytes to form a plaque [75-77]. Subsequently, there is augmented EC barrier dysfunction and vascular smooth muscle cell proliferation eventually leading to plaque rupture and thrombosis [78].

HA regulates EC barrier function and atherosclerosis [18, 58, 79, 80]. Nagy et al., demonstrated that inhibition of HA synthesis with 4-methylumbelliferone (4-MU) in pro-atherosclerotic (apoE knockout) mice resulted in glycocalyx damage and accelerated atherosclerosis [58].

The main receptor for HA, CD44, and the HA-binding proteoglycan, versican, are upregulated in atherosclerotic lesions [79, 81, 82]. When CD44 knockout mice are bred with apoE knockout mice, there is a 50-70% reduction in aortic lesions [82]. Since CD44 is expressed in multiple cell types, the role of endothelial CD44 in HA regulation of vascular integrity with atherosclerosis remains to be determined.

Ischemia/Reperfusion

Ischemia/reperfusion injury leads to tissue damage caused when a blood supply is returned to a tissue/organ after a period of disrupted blood flow (ischemia) [83-85]. Reperfusion of previously ischemic tissue induces reactive oxygen species (ROS) production, activates

inflammatory and blood coagulation cascade responses and increases microvascular permeability [84]. Ischemia/reperfusion is important in numerous processes including cardiac arrest, stroke and organ transplantation [84].

Ischemia/reperfusion injury can stimulate shedding of the glycocalyx [12]. Although the exact mechanism triggering ischemia/reperfusion glycocalyx disruption is unknown, it is believed that increased free radical production along with TNF- α and mast cell degranulation may combine to induce shedding and enzymatic degradation of the glycocalyx [10, 51, 52, 86]. In addition, HA and CD44 expression are upregulated in the microvascular endothelium during ischemia/reperfusion [87, 88].

Toll-like receptors (TLR) have been implicated in the pathology observed in ischemia/reperfusion [89-93]. TLR2 and TLR4, expressed in many cell types including EC, can bind hyaluronan fragments [20, 94]. Zanotti et al., demonstrated that treatment of human pulmonary microvascular EC monolayers with a competitive TLR4 inhibitor protected against simulated ischemia/reperfusion-induced actin cytoskeletal reorganization and gap formation [95]. However, the role of TLRs in HA regulation of vascular integrity during ischemia/reperfusion remains to be determined.

Diabetes

Diabetes refers to a group of metabolic diseases involving hyperglycemia, either caused by insufficient insulin production (Type 1 diabetes) or because of decreased cellular responses to insulin referred to as insulin resistance (Type 2 diabetes) [96, 97]. Hyperglycemia damages vascular integrity through direct and indirect effects resulting in EC barrier disruption [45, 46, 97-99].

Hyperglycemia has also been shown to stimulate shedding of the glycocalyx [45, 46, 100]. Increased levels of plasma HA and decreased glycocalyx volume were observed in healthy volunteers following a 6 hour glucose infusion [45]. Increased plasma concentrations of HA and hyaluronidase have also been detected in type 1 diabetes patients compared to matched controls [80, 100]. In addition, administration of HMW-HA reduced the pathology observed in diabetic (Cg-m+/+Lepr(db)) mice [101].

CD44 (HA-binding receptor) plays a substantial role in diabetes. In animal models of diabetes, CD44 expression is increased [101, 102]. In the transfer model of NOD mice (Type 1 diabetes model), treatment of mice with CD44 antibodies or hyaluronidase induced resistance to insulin-dependent diabetes mellitus (IDDM) [103, 104].

Sepsis

Sepsis refers to a systemic microbial infection characterized by inflammation, activation of the blood coagulation cascade, blood stagnation and clot formation leading to hypoxia and organ failure [11, 105]. Recently, defects in EC barrier function have been suggested as a causative factor in sepsis pathology [106].

Systemic inflammatory response syndrome (SIRS) and sepsis promote damage to the glycocalyx resulting in increased circulating levels of HA, increased inflammatory response and interstitial edema [107]. Increased levels of glycocalyx components in the blood positively correlated with mortality in these conditions [11, 107].

Toll-like receptors (TLR) are crucial in the host response to sepsis [108-112]. TLR sense exogenous and endogenous danger-associated molecular motifs called pathogen-associated molecular patterns (PAMPs) [113-115]. EC mainly express TLR2 and TLR4 [116, 117]. Besides bacterial lipoprotein and lipopolysaccharide, TLR2/4 can bind to a variety of other PAMPs including hyaluronan fragments [20, 94]. Interestingly, Muto et al., 2009 demonstrated that 200-500 kDa HA and CD44 suppress TLR4-mediated septic responses in mice [118].

Acute lung injury

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are the leading causes of death in pediatric and adult critical care patients with a mortality rate of ~40% [119, 120]. An important feature of ALI is endothelial barrier disruption resulting in leakage of fluids, proteins and inflammatory cells into alveoli with consequent pulmonary edema [121].

The main receptor for HA in EC, CD44, is a type 1 transmembrane glycoprotein that undergoes alternative exon splicing between exons 5 and 15 leading to a tandem insertion of one or more

variant exons (v1-v10, or exons 6 through exons 14) within the membrane proximal region of the extracellular domain [122, 123]. We have demonstrated that human pulmonary microvascular EC express the CD44 isoforms, CD44s (standard form) and CD44v10 [18]. *In vitro* models of pulmonary EC barrier function indicate that HMW-HA (~1 million Da) activates CD44s signaling and promotes barrier enhancement through its interaction with the S1P₁ receptor and activation of Rac1 signaling leading to cortical actin formation while HA fragments (~2.5 kDa) activate CD44v10 signaling and induce barrier disruption via the S1P₃ receptor and RhoA-mediated actin stress fiber formation [18] (**Figure 3**).

We have demonstrated that targeted deletion of CD44 in the mouse pulmonary vasculature increases basal leakiness in the lungs [4]. Further, intravenous administration of HMW-HA protects against pulmonary vascular leakiness in a CD44- and caveolin-1 dependent manner in a mouse model of LPS-induced ALI [4].

Lipopolysaccharide (LPS) is a potent endotoxin from Gram-negative bacteria that, when administered intratracheally, produces an inflammatory reaction which includes disruption of the EC barrier and consequent leakage of fluid, protein and immune cells into lung airspaces [16, 124, 125]. Recently, it has been demonstrated by our laboratory and others that CD44 knockout mice have increased BAL protein and HA concentration and exaggerated inflammatory cell recruitment of both macrophages and neutrophils with LPS-induced lung injury [6, 126]. CD44 knockout mice also have increased NF- κ B nuclear translocation and cytokine production [126]. These data suggest that CD44 limits the *in vivo* response to LPS and prevents excessive tissue damage.

Toll-like receptor 4 (TLR4) is expressed in multiple cell types including EC and can bind hyaluronan fragments in addition to other ligands including LPS [94, 124]. Inhibition of TLR4 in animal models and TLR4 loss-of-function mutations in humans protect against LPS-induced lung injury [111, 127, 128]. Interestingly, CD44 deficient mice have decreased expression of negative regulators of TLR including IL-1R-associated kinase M (IRAK-M), Toll-interacting protein (Tollip) and TNF α -induced protein 3 (A20) [129].

Although mainly produced in the liver, we and others have demonstrated that the pulmonary endothelium expresses the extracellular HA-binding serine protease, HABP2, which is upregulated with lung injury [130-132]. HABP2 promotes LPS- and LMW-HA-mediated human pulmonary endothelial cell barrier disruption through a mechanism that involves protease-activated receptors (PAR) [130]. Conversely, HMW-HA inhibits HABP2 activation (**Figure 3**). We determined the contribution of vascular HABP2 to lung injury in mice by inhibiting HABP2 through intravenous administration of HABP2 siRNA and observed attenuation of LPS-induced acute lung injury [130]. In addition, vascular inhibition of HABP2 expression attenuates another mouse model of lung injury with pulmonary vascular hyper-permeability, ventilator-induced lung injury, demonstrating an important role of HABP2 in the regulation of vascular integrity [130].

Concluding remarks

Hyaluronan (HA) plays a crucial role in the maintenance and enhancement of vascular integrity. HA maintains vascular integrity through endothelial glycocalyx modulation, caveolin-enriched microdomain regulation and interaction with endothelial HA binding proteins [4, 18, 28, 130]. Defects in vascular integrity are a causative factor in several disease processes including atherosclerosis, ischemia/reperfusion, tumor angiogenesis, cancer metastasis, diabetes, sepsis and acute lung injury [1, 2, 106]. In disease states such as diabetes and sepsis, increase hyaluronidase activity and reactive oxygen species (ROS) generation which break down HMW-HA to LMW fragments causing damage to the endothelial glycocalyx [17, 20, 80, 107]. HA fragments can activate specific HA binding proteins upregulated in vascular disease including CD44, HABP2, TLR2 and TLR4 to promote actin cytoskeletal reorganization and inhibition of endothelial cell-cell contacts [18, 20, 94, 130, 133](**Figure 3**). CD44 also regulates the EC barrier-enhancing ability of other agents including hepatocyte growth factor (HGF) [6]. In addition to EC, vascular integrity can be regulated by other cell types including vascular smooth muscle cells and pericytes which are beyond the scope of this review. Further, disruption of vascular integrity is crucial for other disease processes including tumor angiogenesis and cancer metastasis. HA plays an important role in these

processes and is described elsewhere [122, 134-141]. The ability of exogenously administered HMW-HA to restore damaged glycocalyx function and enhance EC barrier integrity make it a novel potential therapeutic strategy for diseases with defects in vascular integrity [4, 17, 142, 143].

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Disclosures

Dr. Lennon has no conflict of interest. Dr. Singleton is a provisional patent holder involving applications of hyaluronan with the University of Chicago and has not received any financial gain.

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