REVIEW

Endothelial Glycocalyx Impairment in Disease

Focus on Hyaluronan Shedding

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Hyaluronan (HA) is a ubiquitous glycosaminoglycan of the extracellular matrix. It is present in the endothelial glycocalyx covering the apical surface of endothelial cells. The endothelial glycocalyx regulates blood vessel permeability and homeostasis. HA plays a central role in numerous functions of the endothelial surface layer, protecting the endothelial cells, regulating the barrier permeability, and ensuring mechanosensing, which is essential to nitric oxide production and flow-induced vasodilation. During acute injury, inflammatory conditions, or many other pathologic conditions, the endothelial glycocalyx is damaged, and its degradation is accompanied by shedding of one or more glycocalyx components into the blood. Syndecan-1, heparan sulfate, and HA are the main components whose shedding has been claimed to represent the endothelial glycocalyx state of health. This review focuses on endothelial glycocalyx HA and highlights its key roles in the functions of the endothelial glycocalyx, its shedding in several pathologic conditions such as sepsis, diabetes, chronic and acute kidney injury, ischemia/reperfusion, atherosclerosis, and inflammation, which are all accompanied by increased circulating HA levels. Plasma/serum HA level is becoming recognized as a biomarker of endothelial glycocalyx damage in select pathologies. Hyaluronidase, the main HA-degrading enzyme, and its involvement in the impairment of endothelial glycocalyx are also addressed. (*Am J Pathol* 2020; 190: 768–780; <https://doi.org/10.1016/j.ajpath.2019.11.016>)

In recent decades, the layer that covers the internal surface of blood vessels, on the apical surface of endothelial cells (ECs)—called endothelial glycocalyx (EG)—became increasingly worthy of interest. The EG is a major regulator of endothelial function, and its integrity is crucial to prevent the vascular complications arising from various pathologies.¹ The EG is principally composed of glycosaminoglycans (GAGs) and proteoglycans (PGs). The EG is a fragile structure that is readily damaged in numerous inflammatory and ischemic injuries, leading to endothelial dysfunctions characterized by enhanced vascular permeability, impaired vasodilation, increased leucocyte–endothelium interactions, thrombosis, and vascular inflammation.² EG deterioration is considered an early step in the onset of basically all chronic vascular complications, at the forefront of which

lies diabetes mellitus (DM). EG deterioration can be detected using the plasma levels of two well-established biomarkers, syndecan-1 (SYND1) and heparan sulfate (HS).³ This review focuses on the shedding into the plasma of a third, critical EG component, that is, the noncovalently bound GAG hyaluronan (HA). **Table 1** lists the main pathologic situations known to be related to EG degradation and HA shedding that are discussed in this review.^{4–15} Other EG-degrading pathologies not discussed in this review are hypertension, viral infections, and traumatic brain injury.

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EG and Its Composition

The word *glycocalyx* was invented in 1963 to describe the newly discovered polysaccharide-rich coat located on the external side of the plasma membrane of muscle cells, epithelial cells, and ECs.¹⁶ Since then, it has been mostly (but not exclusively) used to designate the EG lining the inner surface of the blood vessel walls. The EG covers the apical surface of ECs floating in the lumen of blood vessels throughout the body. An EG has also been observed in lymphatic vessels.¹⁷ Because the composition of the lymphatic EG remains unexplored, it will not be elaborated on in this review. *In vivo*, the vascular EG represents a dynamic layer, which is sometimes called the endothelial surface layer (ESL) with reference to plasma components that integrate the EG. This fragile extracellular structure is a porous, hair-like, regularly organized coat, whose thickness is estimated as 0.5 to 5 μm under physiological conditions. This wide range is likely artifactual because it depends on the fixation method (or absence thereof), the dye, and the observation method (optical or electronic microscopy, or indirect methods estimating EG thickness).¹⁸ Nevertheless, it is likely that the thickness and structure of the EG vary across different species, organs, vascular beds, and blood flow rates.¹⁹ Suffice it to say that most studies explore relative changes in EG thickness or composition and that absolute values should not be the main focus of discussion.

EG is set up around membrane-bound PGs, that is, glycoproteins wearing sialic acids, glycolipids, and GAGs in various amounts.²⁰ The main PGs in the EG are syndecans: SYND1, the most widely studied, SYND2, and SYND4. Glypican-1 is the other main EG PG. Syndecans integrate the plasma membrane through a transmembrane domain, whereas glypicans hold onto lipid rafts via a glycosylphosphatidylinositol anchor.²¹ The core proteins of PGs covalently bind several GAGs, mainly HS, chondroitin sulfate, and to a lesser extent, dermatan sulfate and keratan sulfate. Conversely, another GAG, HA, integrates the EG without any covalent binding. *In vivo* examination of the EG has consistently shown two EG layers: a dense inner layer and a sparser outer layer. The inner layer, which forms a primary selective barrier to plasma macromolecules, associates with the plasma membrane and is rich in PGs. These proteins wear HS mostly at their top, projecting this GAG into the outer layer while HA and chondroitin sulfate are

concentrated in the inner layer. The outer layer is mainly composed of GAGs and plasma proteins; its main purpose seems to prevent unexpected blood cell attachment to the EC.²¹ The abundance of HA in the inner layer of the EG endows this layer with other key functional properties which are described below.²¹ *In vivo*, plasma proteins such as albumin, antithrombin III, extracellular superoxide dismutase, lipases, growth factors, and chemokines penetrate and loosely associate with the EG network of GAGs and PGs.¹⁸ In this way, these plasma proteins gain additional contact time with the EC.

Focus on HA

Although HA accounts for only 5% to 20% of the total GAG content of the EG, it is implicated in several key functions of the ESL.²¹ HA is a ubiquitous GAG of all extracellular matrices.²² Its nature (simple repetition of the disaccharide units *N*-acetylglucosamine and glucuronic acid; no sulfate) makes it a long, unbranched, highly anionic polymer able to contact several molecules at the same time while repulsing most cell surfaces due to their negative charges. HA is mainly synthesized as a high molecular weight (HMW) polymer with a molecular mass ranging from 1000 to 6000 kilodaltons (kDa). It is also present in body fluids and tissues as fragments of either medium (250 to 1000 kDa) or smaller (10 to 250 kDa) size. The latter is often called low molecular weight (LMW) HA.²³ HA catabolism and fragmentation result from enzymatic or nonenzymatic degradation. In somatic tissue, enzymatic degradation is principally due to hyaluronidase-1 (Hyal-1) and hyaluronidase-2 (Hyal-2), the main hyaluronidases in mammals. Hyal-1 and Hyal-2 degrade HA into fragments of 2 to 6 disaccharides, and of 20 kDa, respectively.^{23,24} Nonenzymatic degradation of HA mostly results from oxidation, especially in the presence of reactive oxygen species (ROS), including the superoxide anion, hydrogen peroxide, nitric oxide, and peroxynitrite.^{24,25} This route predominates in inflammation and cancer.

The main role of HMW-HA is the assembly and structure of the extracellular matrix, where its large size serves as a scaffold. HA contributes to the resistance to compression of specialized matrices such as cartilage. At physiological pH, soluble HA adopts a random ribbon structure reinforced by internal hydrogen bonds and interactions with the solvent. It therefore occupies a large hydrodynamic volume that allows retention of water molecules, promoting hydration (skin), lubrication (synovial fluid), and filling of extracellular spaces. HA has a high viscosity, and its elastic properties are essential to the protection of cartilage and vitreous humor of the eyes, among others.²⁶ HA tertiary structure confers selective permeability: small molecules such as water, electrolytes, and nutrients can penetrate by diffusion or hydrodynamic flow through the domain created by HA chains, whereas larger molecules such as proteins are

Table 1 Main Pathologic Situations Related to EG Degradation and HA Shedding

Pathological alterations
Systemic or local inflammation ^{4,5}
Sepsis ^{6,7}
Hyperglycemia and diabetes mellitus ^{8,9}
Ischemia—reperfusion injury ^{10,11}
Chronic and acute renal disease ^{12,13}
Atherosclerosis ^{14,15}

partially or completely excluded. These properties are important to all forms of glycocalyx.

In general, HA expression correlates with tissue expansion and cell motility. In particular, HMW-HA can form a mantle (coat) around mesodermal cells and fibroblasts, as observed using electronic microscopy or histological staining.²² This HA-rich coat embodies HA's ability to exclude other cells or particles. At the biological level, this coat allows the cell to interact more selectively with its environment and protect itself against immune cells and viral infections. This coat is sometimes called cellular glycocalyx. The EG is a specialized version of the cell coat or glycocalyx.

In general, HMW-HA predominates under physiological (healthy) conditions, displaying anti-inflammatory, anti-angiogenic, and immunosuppressive properties, whereas HA fragments predominate under pathologic conditions and are highly inflammatory, angiogenic, and stimulant to the immune system.²⁷ HA plays a critical role during inflammation. Several inflammatory cytokines induce its production and, once fragmented, notably by ROS, HA in turn induces the synthesis of cytokines, intensifying inflammation.²⁸ HA, likely in the form of HMW-HA, can form cable structures that are modified by tumor necrosis factor-stimulated gene 6 protein (TSG-6). TSG-6, stimulated by inflammation or high glucose levels, transfers heavy chains from the inter-alpha trypsin inhibitor onto HA. These special HA cables become adhesive for inflammatory cells, preventing them from binding to the endothelium or other structures.²⁹ On the other hand, small HA fragments stimulate proliferation and migration of ECs, initiate angiogenesis,³⁰ and increase expression of the typical markers of endothelial dysfunction such as inter-cellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1).³¹ Similarly, LMW-HA, through interaction via the common HA receptor CD44, enhances actin stress fiber formation, leading to disruption of the EC barrier.³² In summary, *in vitro*, HMW-HA is a protector of the endothelium, whereas lower-size HA has detrimental effects.

HA, a Key Actor of the EG

HA integrates the EG through both electrostatic interactions and binding to its main receptor, CD44, located at the plasma membrane.³³ The fact that CD44 is a transmembrane protein does not prevent HA from floating away from the EC apical surface into the ESL, due to the length of the intact HA molecule. HA can also remain attached to HA synthases during its synthesis, including in ECs.³⁴ Although HA synthases are located, in all their host cells, at the inner face of the plasma membrane, they extrude the newly synthesized HA polymer toward the extracellular space through a pore.³⁵ How long the HA molecule remains attached to the synthase pore while floating on the external cell surface is not known. Other ways allowing HA to attach to the EG

could be through hydrophobic patches with other GAGs such as chondroitin sulfate,³⁶ other HA-binding proteins such as versican or hyaluronan receptor for endocytosis (stabilin-2) besides CD44,^{37,38} or self-assembly into a fibrous network.³⁹

In the vasculature, HA has been unequivocally shown to play a fundamental role in the maintenance of vascular integrity. It is implicated in most EG functions regulating vessel homeostasis.⁸ The main functions attributed to EG HA have been discovered by injecting different forms of hyaluronidase active at neutral pH into the circulation. Hyaluronidase injection was thus used to validate the assessment of EG systemic volume by dextran clearance, EG size in microcirculation by sidestream dark field imaging,⁴⁰ and EG stiffness by reflectance interference contrast microscopy⁴¹ or by atomic force microscopy.^{42,43}

The functions of EG HA can be summarized as follows:

Under physiological conditions, HA contributes to the EG thickness. GAG-degrading enzymes have been repeatedly used to evaluate EG thickness *in vitro* and *in vivo*. The results of these experiments are highly variable. This may be due to the nature of the vessels studied and the conditions of the treatment (nature of the enzyme, and amount and duration of exposure). For example, bacterial heparinase, chondroitinase, and hyaluronidase from bovine testes, injected into rat mesenteric post-capillary venules, induced a 43%, 34%, and 26% decrease in EG thickness (visualized using intravital microscopy), respectively.²⁰ EG thickness of rat myocardial capillaries treated with bovine testicular hyaluronidase and observed through electronic microscopy was reduced by 58%,⁴⁴ whereas the same treatment of mouse carotid arteries observed using two-photon laser scanning microscopy reduced EG thickness by 25%.⁴⁵ *In vitro*, bovine testicular hyaluronidase produced a modest 15% decrease of EG thickness in bovine lung microvascular ECs,⁴⁶ as did *Streptomyces hyalurolyticus* hyaluronidase in the same model,⁴⁷ but in rat fat pad ECs the bacterial hyaluronidase reduced EG thickness by 40%.⁴⁸

However, the role of endogenous hyaluronidases or HA synthases in EG HA homeostasis has not been proved as of yet. This element is discussed in the context of specific pathologies.

EG HA is an integral part of vessel permeability through repulsing or slowing down protein and macromolecule passage. In practice, a small protein-free zone is created in the deep EG layer, with an oncotic gradient directed toward the lumen of the vessel, limiting water efflux out of the bloodstream. That gradient is reduced when HA is eliminated by *in vivo* enzymatic degradation.⁴⁹ More recently, studies using optical and fluorescence microscopy reported that HA and chondroitin sulfate present in the denser layer of EG are responsible for the selective permeability of the EG, whereas HS, concentrated in the upper layer of the EG, helps maintain the overall EG structure.²⁰ Accordingly, hyaluronidase treatment induced diffusion of a fluorescein dye into the sublayer of the EG, whereas heparinase (an

exogenous enzyme degrading HS) did not. Recently, the role of HA in glomerular fenestrated endothelium has been highlighted using an endothelium-specific and inducible deletion of hyaluronan synthase-2 in mice. Loss of EG HA in glomeruli resulted in EG thickness decrease, capillary destabilization with loss of endothelial fenestrations, and development of albuminuria.⁵⁰

HA is probably the main actor in EG's mechanosensory effect. The EG translates signals from the blood flow (eg, laminar versus turbulent flow) to the EC cytoskeleton. The use of isolated porcine superficial femoral arteries or canine femoral arteries incubated with hyaluronidase and subjected to flow has demonstrated the role of HA in flow-mediated NO production and vasodilation.⁵¹ Cultured ECs harvested from bovine thoracic aortas or rat brain microvessels and treated with hyaluronidase have been used to confirm the mechanosensory role of HA in flow- or stretch-induced NO synthesis.^{52,53} On the other hand, atomic force microscopy failed to demonstrate HA involvement in NO production.⁵⁴ Finally, HA has also been described as a cushioning layer able to distribute applied forces over the whole EG structure.⁴¹

Under pathologic conditions, HA helps EG restrict leukocyte and platelet adhesion to the endothelium and thus regulates inflammation and thrombosis.^{14,55} This was shown using hyaluronidase infusion, which stimulated macrophage accumulation—likely due to higher monocyte infiltration—in the perivascular space in a rabbit model of femoral artery ligation.⁵⁵

EG HA is also part of capillary function: the EG damages following hyaluronidase treatment revealed a reduction of functional capillary density in whole body hamster microcirculation.⁵⁶

Despite a thicker EG consistently reported in large vessels versus microcirculation,^{1,57} hyaluronidase treatment seemed to confirm HA involvement in EG functions independently of vessel size. For instance, EG degradation of rat coronary artery of perfused hearts with hyaluronidase lead to myocardial edema,⁴⁴ and hyaluronidase treatment of hamster cremaster microcirculation, mice retinal arterioles, and rat post-capillary venules altered their selective permeability barrier.^{20,49,58}

Several EG-Degrading Pathologies Involve HA

In a set of diseases and pathologic conditions described below, EG degradation is reported as an early and key step in endothelial dysfunction and vascular complications. This EG damage generates fragments that are released into the plasma, such as HA, HS, or SYND1. These elements are used as biomarkers of EG shedding (Figure 1). Four main factors have been implicated in EG degradation: heparanase (the endogenous enzyme degrading HS) and matrix metalloproteases (MMPs)—these two families of enzymes are called sheddases because they directly cleave EG

components and induce their shedding into the plasma—ROS, and tumor necrosis factor (TNF)- α . These four factors often work in concert. In DM, advanced glycation end-products (AGEs) may also act directly on the EG. Finally, Hyal-1 was recently added to the list of EG sheddases.^{8,59}

With regard to the sheddases, heparanase, which selectively cleaves HS attached to core PGs, may be the most ubiquitous. Heparanase is secreted by numerous cell types including macrophages, podocytes (during diabetes), or mast cells.^{60,61} MMPs, which cleave PGs (eg, SYND1, SYND4) directly off the EC membrane, are also implicated in EG degradation.³ Among them, MMP-2, MMP-9, MMP-13, and MMP-15 have been pointed out.⁶² General inhibition of MMPs using doxycycline or zinc-chelating hydroxamic acid protected against EG loss in rat mesentery post-capillary venules challenged with the chemo-attractant f-Met-Leu-Phe.⁶³ The involvement of hyaluronidases was suspected, but not confirmed,^{15,60,64} until a recent publication showed the role of Hyal-1 in HA shedding during early experimental diabetes.⁵⁹

TNF- α is also a recognized actor in EG degradation. This cytokine forces leukocytes to release free radicals and/or proteases, and mast cells to liberate histamine, cytokines, and numerous enzymes, including heparanase.⁶⁵ Furthermore, TNF- α itself is able to activate MMP-9 and heparanase.^{3,62} ROS, a systematic consequence of hyperglycemia, inflammation, and ischemia/reperfusion (I/R), are a main source of nonenzymatic EG degradation. They are able to depolymerize GAGs and particularly HA.^{4,25,66} They can also activate heparanase and MMPs.³

Thus, the main mechanisms of HA degradation in pathologic conditions include its breakdown into smaller fragments by ROS and hyaluronidases, with the addition of AGEs in DM. This review focuses on HA and hyaluronidase involvement in EG deterioration in human diseases and their animal models, with the hope to generate increased interest in this key EG component. In some instances, knowledge on the role of heparanase or other sheddases is mentioned if relevant. Figure 2 summarizes the mechanisms of EG HA shedding reported in various diseases, and the potential use of plasma HA level as a biomarker assessing the endothelial glycocalyx state of health.

Sepsis

EG Degradation in Sepsis

Sepsis, or systematic inflammation, sometimes leading to death, follows several common pathophysiological courses including excessive activation of the immune system and increased microvascular permeability. The latter results in plasma fluid leakage, hypovolemia, interstitial edema, and multiorgan failure. EG degradation has often been reported during sepsis, both in clinical studies and in animal models.^{65,67,68} In septic mice, for instance, EG thickness measured by intravital microscopy decreased by up to

Figure 1 Schematic representation of the endothelial glycocalyx covering endothelial cells in the lumen of the vessels, under physiological conditions (left of the **white dashed line**) and during various diseases inducing shedding of its components (right of the **white dashed line**). Hyaluronan is shown in light green; heparan sulfate in blue; syndecan-1 in yellow.

66%.⁶⁹ In healthy subjects injected with low-dose endotoxin, a decreased sublingual EG thickness was measured using orthogonal polarization spectroscopy imaging, in parallel with an increase in HA plasma level.⁷⁰ EG degradation represents one of the earliest and most significant sites of injury during sepsis. A thinner and sparser EG is related to vascular hyperpermeability and resulting edema, hypovolemia, vasodilation troubles, leukocyte attraction, platelet aggregation, and lung injury.^{3,71} Several of these dysfunctions have been linked to HA degradation.

Plasma and Urine HA as Biomarkers of Glycocalyx Shedding in Sepsis

In sepsis, increases in plasma SYND1 and HS are accepted markers of EG shedding,³ which strongly correlate with disease severity.⁷² Besides these well-established markers, plasma HA was also found to be elevated in parallel with disease severity in several clinical studies.^{72–74} For instance, in patients with sepsis due to pneumonia, plasma HA concentration peaked at 3 hours and stabilized till 24 hours, whereas plasma SYND1 increased over time (0 to 24 hours).⁶ HA, like SYND1, was a significant predictor of survival in one study that included measurements at several time points,⁷² but not in another study with admission measurements only.⁷⁴

Animal models of sepsis provided similar results and allowed a comparison of EG degradation and rise in biomarkers. For instance, cecal ligation and puncture, or inhaled lipopolysaccharide induced a decrease in EG thickness already after 4 and 8 hours.⁵ In parallel, plasma HA, SYND1, and HS increased over the first 24 hours. In

another study, plasma SYND1 and HA were increased 8 hours after lipopolysaccharide-induced pulmonary inflammation.⁵ A rat model of sepsis highlighted the time course of SYND1 and HA shedding⁷: plasma HA peaked 3 hours after lipopolysaccharide injection and then regressed, whereas the increase in SYND1 was more sustained. This study suggested an early degradation of GAGs (HS and HA) from the EG followed by disruption of PGs. Association of EG degradation with inflammation was observed in a dog septic shock model in which plasma SYND1 and HS correlated with IL-6 and TNF- α 64.⁷⁵

HA and HS can also increase in urine, as shown in sepsis patients within 24 hours of admission, in comparison with trauma patients admitted to surgical intensive care unit.⁷³ In that case, urine HA and HS associated with septic shock mortality. These increased levels probably reflect renal EG degradation since glomerular filtration of systemic HA and HS should remain limited even with reduced renal function.

Mechanism of EG Degradation and Involvement of Hyaluronidase in Sepsis

ROS and proinflammatory cytokines such as TNF- α and IL-1 β are considered the main actors in EG degradation in sepsis.⁶⁵ Both mechanisms activate the sheddases heparanase and MMPs. In sepsis, TNF- α plays a critical role by inducing EG degradation and activating heparanase.^{65,70} MMP-15, which cleaves the HA receptor CD44 ectodomain, also actively participates in EG degradation.⁷⁶ Recently, angiopoietin-2 was also pointed out as a key mediator of EG degradation in a mice model of sepsis and

Sepsis	Kidney injury	Diabetes	Ischemia-reperfusion	Atherosclerosis	Pathologies
		ROS			EG HA Shedding Mechanisms
		AGEs		Disturbed flow	
	Hyaluronidase			Hyaluronidase	
HA	HA	HA	HA		HA as Plasma Biomarker

Figure 2 Mechanisms of HA shedding from endothelial glycocalyx (EG) as reported in various diseases, and evidence for the use of HA plasma level as a biomarker to evaluate the endothelial glycocalyx state of health. **Hatched boxes** represent potential hyaluronidase involvement. AGEs, advanced glycation end-products; HA, hyaluronan; ROS, reactive oxygen species.

on cultured human umbilical vein endothelial cells. Angiopoietin-2 is secreted by ECs during inflammation and prevents angiopoietin-1's beneficial properties toward the EG. Exogenous infusion of angiopoietin-1 was reported to increase EG depth, decrease water permeability, and increase albumin retention in frog mesenteric microvessels.⁷⁷ Moreover, angiopoietin-1 restored the EG in glomeruli of diabetic rats.⁷⁸ In the same way, inhibition of angiopoietin-2 reduced EG degradation and improved animal survival during sepsis.¹⁹

Is there a role for hyaluronidase in sepsis-induced EG degradation? Such a role has long been suspected and often proposed, considering the strong evidence for HA shedding.^{60,71} However, experimental evidence remains scarce as of yet. In a mouse model of sepsis, serum hyaluronidase activity (due to Hyal-1) did increase 24 hours after cecal ligation and puncture.⁵ Also, ECs respond to endotoxin stress by releasing Weibel-Palade bodies and secretory lysosomes containing several enzymes such as sphingomyelinase, glycohydrolases, cathepsins, and HS sulfatase.⁶⁸ Hyal-1 is present in endolysosomal compartments following a secretion–recapture event in macrophages,⁷⁹ and also is actively endocytosed from the plasma to liver sinusoidal ECs.⁸⁰ Therefore, it may very well be a part of this exocytotic process and trigger HA release from the EG. It is not inconceivable, but unlikely, that bacterial hyaluronidase activity also plays a role in some rare septic situations.⁸¹ HA can also be degraded directly by ROS production.

Ischemia/Reperfusion

Sepsis is characterized by inflammation and disrupted organ perfusion. The same mechanisms are in play during organ or whole-body ischemia followed by reperfusion. Therefore, it does not come as a surprise that similar observations on the EG have been made in sepsis and I/R injuries.⁸² These injuries and resulting EG damage accompany, for example, thoracic surgery (cardiopulmonary bypass or repair of aortic

aneurysms) and coronary artery bypass surgery with or without cardiopulmonary bypass.^{10,83} Global or regional I/R also occurs during deep hypothermic cardiac arrest¹¹ and transplantation of rewarmed kidneys.⁸² EG shedding has been confirmed in several animal models of I/R, including the lungs,⁸⁴ liver,⁸⁵ heart,⁸² kidneys,⁸⁶ and mesenteric area.⁸²

HA as a Biomarker of EG Shedding in I/R

Elevation of plasma SYND1 and HS has been observed in all these clinical and experimental I/R investigations.^{11,83} In a few cases, plasma HA was also measured and reported to be elevated, notably in post-cardiac arrest syndrome,¹¹ coronary artery bypass surgery,¹⁰ and renal I/R injury.⁸⁶ In a model of rat mesenteric I/R, infusion of HMW-HA (800 to 1200 kDa) was effective in preventing (if infused before I/R) or reversing (if infused during the first 5 minutes of reperfusion) the effect of I/R on the EG in mesenteric capillaries and post-capillary venules. This protective effect was attributed to the antioxidant properties of HA and its ability to reconstitute the degraded EG.⁸⁷

Mechanisms of EG Alteration during I/R and Hyaluronidase Involvement

Although the exact mechanism responsible for EG disruption during I/R remains to be clarified, involvement of free radical production, mast cell degranulation, activated inflammatory cells, and blood coagulation cascade responses has been suggested.^{60,87} The main culprit is undoubtedly the abundance of reactive oxidative and nitrosative species associated with I/R. These reactive species originate from various cell sources including ECs. During reperfusion, for example, functional endothelial nitric oxide synthase (eNOS) dimers become uncoupled and induce the production of superoxide anion. The role for xanthine-oxidoreductase, also generating superoxide anion, has been confirmed.⁸⁷ Oxidative damage to all GAGs has been linked to almost all biologically relevant radical (hydroxyl radical, carbonate radical, superoxide anion) and

nonradical (hypochlorous acid, peroxynitrite ion/peroxynitrous acid) oxidants.⁸⁸ *In vitro* investigation of HA or its chloramide derivate (chloramide-HA) generated during inflammation -corroborated HA fragmentation by oxidative radicals, especially carbonate radical and hydroxyl radical. Their effects on HA are similar to those of hyaluronidase. Fragmentation of HA is also mediated by peroxynitrous acid and peroxynitrous acid-derived hydroxyl and hypochlorous acid/hypochlorite ion. Chloramide-HA appears to be even more susceptible to fragmentation than chloramide-HS.⁸⁸ EG loss can be prevented by neutralizing superoxide anion and hydrogen peroxide using SOD and catalase during I/R injury.⁸⁷

Involvement of mast cells, recruited and activated neutrophils, and activated platelets is also relevant in the induction of EG shedding during I/R, as these cells can release various proteases and enzymes, such as heparanase or cathepsin B, known to degrade EG components.⁸⁹ Neutrophilic granulocytes adhering to the endothelium contain many proteolytic enzymes (serine proteases, elastases) that can be spread out along the endothelium⁶⁰ and probably take part in cleavage of core proteins of the PG family, accounting for HA shedding through CD44 ectodomain cleavage.³³ Upon activation, these neutrophils produce reactive species and thus reinforce oxidative or nitrosative stress.⁸⁶ Leukocytes have not been demonstrated to express hyaluronidase activity. ROS are probably the major, if not sole, mechanism by which leukocytes degrade HA.^{90,91} However, these studies have not been repeated using more recent methods of detection; thus, the presence of hyaluronidase activity in activated leukocytes cannot be excluded at this stage.

In addition to enzymatic degradation, atrial natriuretic peptide (ANP) was shown to take part in EG disruption in patients undergoing coronary artery bypass surgery. The elevation of ANP coincided with or even preceded shedding of the EG.¹⁰ Because ANP possesses no enzymatic activity able to induce this shedding, the authors proposed that the peptide could activate some unidentified protease. The effect of ANP on hyaluronidase activation could be explored in the future. Expression of TNF- α was also observed in a murine model of I/R which implicated superoxide anion production; TNF- α can indisputably take part in EG destruction.⁹² Recently, the anaphylatoxin C5a has been implicated in EG shedding during I/R injury. This complement fragment, acting through its own receptor, activates neutrophils to produce reactive oxidative and nitrosative species and to release granular proteases.⁸⁶ During renal I/R, EG degradation was prevented, and kidney function preserved, using an anti-C5 antibody.⁸⁶

On the other hand, involvement of hyaluronidases in I/R injury has not been reported to date.

Diabetes Mellitus

Besides sepsis and I/R injury, DM is the other major pathology implicating EG degradation. In a way, DM is even

more important than sepsis or I/R when it comes to the implication of the EG, because EG defects in DM are early events, may portend more severe vascular complications, and serve as prognostic factors.

EG Degradation in DM

EG degradation during DM has been demonstrated in several animal models of type 1 and type 2 DM (T1DM and T2DM, respectively), as well as in humans.^{8,58,93,94} Both in T1DM patients and during induced acute hyperglycemia, evaluation of EG using intravital microscopy revealed a decrease in its systemic volume, calculated as the difference between the volume of distribution of neutral dextran 40 (penetrating the EG) and plasma volume (plasma macromolecules excluded from the EG).^{9,94} A decrease of EG volume in sublingual and retinal vessels, measured using sidestream dark field imaging and combined fluorescein/indocyanine green angiography, was also observed in T2DM patients and is proposed as a useful clinical measure,⁹³ although it has not been adopted extensively as of yet. In T1DM and T2DM animal models, damage to the EG has been frequently reported. For instance, transmission electron microscopy and intravital microscopy revealed a decrease EG size in retinal vessels of T1DM and T2DM rats and mice,^{58,95} and in brain microvessels of T2DM mice.⁹⁶ Glomerular EG observed in a mouse model of diabetic nephropathy using immunohistochemistry or transmission electronic microscopy also appeared to be reduced in size.⁹⁷ An atomic force microscopy study of the EG in diabetic *db/db* mice highlighted its gradual alteration in early diabetes (11-week-old mice).⁴³ In that study, EG thickness was reduced with diabetes progression and correlated with increasing level of glycated hemoglobin.

Plasma HA as a Biomarker of EG Shedding in DM

Independently of EG investigation, modifications in blood GAG concentrations of DM patients was reported as early as 1973; plasma HA levels were then found to correlate positively with glycated hemoglobin levels.⁹⁸ More recently, HA and SYND1 were both proposed as reliable blood or plasma markers of EG shedding in DM or even acute hyperglycemia.^{9,94} Indeed, during acute hyperglycemia or in patients with T1DM, EG thickness was reduced by 50% to 80%, and plasma HA concentration increased by 30% to 80%.^{9,15,94} This HA shedding was directly connected with increased vascular permeability and albuminuria, overactivated coagulation, and decreased flux-induced vasodilation. Degraded EG coupled with increased plasma HA concentration is also observed in T2DM patients⁸⁰ and in rodent models of T1DM.^{59,65}

Concomitantly with higher plasma HA, plasma hyaluronidase activity was also elevated in DM patients,^{93,94} as expected if Hyal-1 was overproduced and then captured by ECs, resulting in HA shedding.

Mechanisms of EG Alteration during Hyperglycemia, Involvement of Hyaluronidase

Involvement of ROS, AGEs, and activation of the enzymes heparanase and Hyal-1 in EG deterioration during DM has received multiple experimental support. ROS are abundant in diabetes: An overproduction of superoxide anion and resulting increased oxidative stress systematically accompany hyperglycemia. ROS are able to attack the EG through depolymerization of GAGs and more particularly HA.^{4,25,66} Antioxidant treatment can partially avoid EG damage.⁹ AGEs are also responsible for EG damage through HMW-HA degradation.⁹⁹ The role of heparanase is also well described in diabetes-induced kidney damage. Heparanase is secreted by macrophages and endothelin-activated podocytes and seems directly implicated in the process of EG degradation leading to diabetic nephropathy.⁶¹ For instance, reduction of heparanase expression by macrophages using inhibition of monocyte chemo-attractant protein-1 restores the EG in a mouse model of diabetic nephropathy.¹⁰⁰ *In vitro*, ECs cultured under high glucose conditions increase their rate of heparanase secretion.¹⁰¹

The role of Hyal-1 in DM has recently been exposed. Plasma hyaluronidase activity measured *ex vivo* reflects Hyal-1 activity. This activity increased in mice, rats, and humans with DM.^{15,59,64,93,94} Furthermore, our laboratory has shown that streptozotocin-induced Hyal-1 knockout diabetic mice display a thicker EG and are protected from HA shedding, endothelial dysfunction, and microalbuminuria in early DM, when compared with Hyal-1-competent mice. Together, these observations point to Hyal-1 as a major actor in early diabetic vascular (and possibly renal) complications.⁵⁹

Finally, MMP-2 and -9 could also be implicated in EG shedding during DM, for instance, following decreased NO availability.¹⁰² NO has been shown to inhibit MMP-2 and MMP-9 in other pathologies such as myocardial infarction. MMP-9 mediates shedding of SYND4 in glomerular ECs through the inflammatory cytokines IL-1 β and TNF- α .^{62,103}

Atherosclerosis

The advent of EG degradation during DM is intimately linked to the development of vascular complications in the context of atherosclerosis. DM is known to accelerate the development of atheromatosis. However, atheromatosis itself associates with EG deterioration even in the absence of DM.

The development of atherosclerosis follows several steps, including endothelial dysfunction (mainly EC barrier disruption) and accumulation of low-density lipoproteins (LDLs), cholesterol, and monocytes in the subendothelium to form a plaque. Worsening of EC barrier dysfunction and vascular smooth muscle cell proliferation lead to plaque rupture and thrombosis. EG damage has

been reported to be a common initial step in atherogenesis. For instance, EG thickness, observed using electron microscopy, appeared significantly thinner, whereas the intima-media ratio increased, in pro-atherosclerotic apolipoprotein E (*APOE*) knockout mice or transgenic *APOE**3 mice (containing a mutant human *APOE* gene associated with dyslipoproteinemia) fed a high-fat, high-cholesterol diet.^{104,105} The EG, which is highly abundant in the common carotid region exposed to laminar flux and resistant to atherosclerosis, appears sparse or even absent in athero-prone regions such as the internal carotid sinus, where blood flow is turbulent. This thinner EG shows impaired LDL retention resulting in increased LDL accumulation in the intima.¹⁰⁴ EG shedding thus probably plays a role in endothelial dysfunction and inflammation of athero-prone regions of the vasculature.¹⁰⁵ In the balloon injury rat carotid artery model, damage to the EG accompanied increased atherosclerosis-related factors (osteopontin and VCAM-1), dyslipidemia, infiltration of inflammatory cells, and impaired eNOS activity. All these factors appeared to be normalized after EG repair using the EG protector sulodexide.¹⁰⁶ These observations strengthen the link between atherosclerosis and EG destruction. In a large cohort of individuals (>6000), however, a reduced size of EG measured in sublingual capillaries did not correlate with classical atherosclerotic risk factors including age, diastolic and systolic blood pressure, LDL, high-density lipoprotein, triglycerides, and body mass index.¹⁰⁷ Only sex and diabetes were independently associated with EG size. EG thinning may thus be another atherosclerotic factor of its own.

EG degradation is often presented as an initiating step in the process of atherosclerosis, at the top of the waterfall of events leading to endothelium dysfunction. A recent study, in *APOE*/*LDLR* (LDL receptor) deficient mice fed a chow diet, reported that EG damage did not precede endothelial dysfunction but rather coincided with impairment of endothelial-dependent vasodilation, increased endothelial permeability, lower NO production, and endothelial inflammation measured by sVCAM-1, in a vicious circle concept.¹⁰⁸

HA Investigation in Atherosclerosis

Abnormal HA deposition in vessel walls and plaques, as well as tissue HA involvement, has been reported in atherosclerosis.^{109,110} HA, in this case, is a bad actor in disease progression. CD44 is also up-regulated in atherosclerotic lesions, and cross-breeding of proatherosclerotic *APOE* knockout mice with CD44-deficient mice reduced plaque formation.¹¹⁰ However, HA is well known to be involved in EC barrier function, and suppression of HA deposits in vessel walls during atherogenesis did not produce any beneficial effect.¹⁴ On the contrary, inhibition of HA synthesis using 4-methylumbelliferone in proatherosclerotic *APOE* knockout mice accelerated atherosclerosis because of the simultaneous reduction in EG thickness and disappearance of EG HA,

concomitant with an altered relaxation in response to acetylcholine and a rise in plaque areas.

Rather surprisingly, plasma HA measurements have not been reported in the field of atherosclerosis, except when the latter occurs as a complication of DM. EG damage and perturbed HA metabolism have been reported in T1DM patients, as mentioned above. Plasma HA and hyaluronidase increased in these patients and corresponding animal models, and they correlated with carotid intima-media thickness.¹⁵ Based on these observations, altered HA metabolism could be involved in the accelerated atherosclerosis process of DM, but the role of HA in nondiabetic atherosclerosis is less clear.

Mechanism of EG Shedding and Hyaluronidase Involvement in Atherosclerosis

Several factors seem to be involved. Low shear stress due to reduced or turbulent blood flow is linked to endothelial inflammation and atherosclerosis in bifurcation areas of the vascular tree. Indeed, low shear stress has been repeatedly shown to impair EG^{104,111} and in particular to lead to HA degradation in the EG.¹¹¹ Another HA-degrading enzyme, Hyal-2, may be involved in these events: In cultured human umbilical vein endothelial cells, Hyal-2, attached to the external side of the plasma membrane through a GPI anchor, became activated under low shear stress (<5 dyn/cm²) and was responsible for HA degradation in the EG.¹¹² Monocyte adhesion and macrophage infiltration in the vascular wall during atherogenesis can further degrade the EG: Infiltrating macrophages can express elastase and MMPs¹⁰⁶ and increased expression of MMP-9 co-localized with EG shedding and plaque buildup.¹⁰⁵ Various risk factors of atherosclerosis such as hyperglycemia, oxidative stress, smoking, and systemic inflammation could also be added as potential mechanisms involved in EG damage during atherosclerosis.¹¹⁰

Acute and Chronic Kidney Disease

EG Degradation in Chronic Kidney Disease

In line with what has been discussed previously about sepsis, DM, and atherosclerosis (*EG Degradation in Sepsis*, *Ischemia/Reperfusion*, and *EG Degradation in DM*), patients with chronic kidney disease (CKD) develop endothelial dysfunction in the context of widespread vascular complications. A study focusing on the EG reported that CKD is accompanied by increased plasma levels of HA and SYND1, as well as the typical markers of endothelial dysfunction, sVCAM-1, von Willebrand factor, and angiotensin-2.⁴² Similar biomarker observations were made in a rat model of CKD in which aortic EG thickness and stiffness observed through atomic force microscopy were significantly reduced.⁴² Dialysis patients also presented with increased plasma HA and SYND1, in line with deeper penetration of red blood cells into the EG of sublingual capillaries, a sign of damaged barrier function of

the EG.¹² Elevated plasma HA correlated with the duration of dialysis treatment, so possibly with more severe EG damage. A high level of plasma HA was reported in other studies that did not assess EG but defined HA as a predictor of poor survival in dialysis patients.¹¹³ The protective role of EG, particularly HA and chondroitin sulfate, in CKD was confirmed in mice with diabetic nephropathy, in which EG damage and increased albumin permeability following chronic administration of hyaluronidase and chondroitinase could be blocked through vascular endothelial growth factor C treatment.¹¹⁴

Involvement of Hyaluronidase in Acute and Chronic Kidney Disease

Not only HA, but also Hyal-1, activity is increased in the serum of dialysis patients.¹² This combination suggests the involvement of Hyal-1 in EG degradation, although EG damage can also occur during the chronic inflammation and oxidative stress brought by CKD. However, the link between increased plasma HA and EG damage in CKD is compounded by the effect of renal function impairment on HA clearance. Although HA is primarily degraded locally in tissue and in lymph nodes (up to 90% of lymph HA seems to be degraded in the nodes), the remaining, circulating HA is degraded by the liver (85% to 90%) or excreted by the kidney (10%).¹¹⁵ In CKD patients, a close relationship between the degree of glomerular filtration decrease and the increase in plasma HA was observed.¹¹⁶ The kidney presumably filters the LMW fraction of HA.¹¹⁶ Accordingly, the urine of dialysis patients with residual renal function contains only LMW-HA, and 30% of their total plasma HA is accounted for by LMW-HA.¹¹⁷ Conversely, only HMW-HA could be detected in the plasma of healthy subjects.¹¹⁸ In summary, a combination of reduced renal clearance and increased shedding from a damaged EG likely explains the high levels of plasma HA found in CKD and dialysis patients.

Degradation of the EG has often been reported in acute kidney injury (AKI). Recent evidence suggests a key role of EG dysfunction in AKI pathogenesis.¹¹⁹ Increases in plasma SYND1 were reported in AKI following, for example, pediatric cardiac surgery,¹¹⁹ acute decompensated heart failure,¹¹⁹ orthotopic liver transplantation,¹²⁰ leptospirosis,¹¹⁹ and post-cardiopulmonary bypass AKI in adult pigs.¹²¹ Other measures of EG degradation have not been reported in these settings. Serum HA levels, however, may be a better marker of CKD than of AKI. For instance, in patients with high uremia and unknown renal history, a twofold higher HA level was measured in those suffering from progressive CKD compared with those suffering from AKI.¹³ In that study, HA serum levels correlated with proteinuria only in CKD patients, not in AKI patients. However, no direct investigation of the EG was reported. In a mouse model of nephrotoxicity after adriamycin injections, glomerular EG was reduced to 20% of its normal thickness within 6 days. Simultaneously, glomerular EG PGs, the receptor for hyaluronan mediated motility

(RHAMM), and Hyal-2 expression were down-regulated. HA was not directly measured in that study.¹²²

Conclusions

HA is a known component of the EG that is essential to its thickness and flexibility. HA provides the ESL with some of its key properties, especially mechanotransduction, restricted penetration of leukocytes, and selective permeability.

Physiologically, the regulation of the amount and molecular size of HA in the EG seems to depend mostly on hyaluronidases, with Hyal-1 as the main controller. A right balance of HMW-HA and LMW-HA is required to ensure appropriate access of leukocytes and immune cells to the EC barrier without undue inflammatory stress. However, the true importance of EG HA is revealed in several pathologies that primarily damage the endothelium, such as sepsis, I/R, DM, atherosclerosis, and AKI/CKD. In all these diseases, the loss (shedding) of HA contributes to the aggravated pathology.

The mechanisms of EG degradation in these diseases have been ascribed to activation of the sheddases heparanase and MMPs, but also to ROS and TNF- α , depending on the disease type. Hyaluronidase involvement has been much less studied, but closer examination of published evidence reveals that this HA-degrading enzyme can be considered a *bona fide* sheddase. In particular, increased plasma/serum hyaluronidase activity (due to Hyal-1 released from the EG and the liver) is observed in experimental and human DM, in dialysis patients, and in experimental mouse sepsis. Hyal-1 could simply be released from the EC layer during endothelial damage and propagate HA, and thus ESL, degradation. Strikingly, Hyal-1 knockout mice display a thicker EG that partially protects them from the early harms of DM. Increased ROS production is another common factor in pathologic EG HA degradation.

In parallel, shedding of HA from the EG, and increased plasma and urine concentrations of HA, have been progressively recognized as a reliable biomarker of the state of health of the ESL in sepsis, I/R, and DM, besides the more familiar biomarkers SYND1 and HS. Blood/plasma HA is easy to measure using available enzyme immunoassays. During sepsis, increases in circulating HA levels express disease severity and may predict clinical outcome. DM is universally characterized by HA shedding from the EG, possibly at an early stage as shown in animal models and during hyperglycemia in humans. More longitudinal studies are needed to assess the real benefit of measuring plasma HA (as well as plasma hyaluronidase activity or plasma Hyal-1) during the course of DM. Several forms of acute and chronic kidney injury release EG HA along with typical markers of endothelial dysfunction; HA is a candidate marker of CKD. During heart or kidney I/R, including post-cardiac arrest syndrome, coronary artery bypass surgery,

and AKI, assessment of HA shedding may orient the therapeutic approach. Finally, hyaluronidase inhibitors are on the list of novel EG-protecting therapies.

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