



REVIEW ARTICLE

Derangement of the endothelial glycocalyx in sepsis

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To cite this article: Iba T, Levy JH. Derangement of the endothelial glycocalyx in sepsis. *J Thromb Haemost* 2019; 17: 283–94.

Summary. The vascular endothelial surface is coated by the glycocalyx, a ubiquitous gel-like layer composed of a membrane-binding domain that contains proteoglycans, glycosaminoglycan side-chains, and plasma proteins such as albumin and antithrombin. The endothelial glycocalyx plays a critical role in maintaining vascular homeostasis. However, this component is highly vulnerable to damage and is also difficult to examine. Recent advances in analytical techniques have enabled biochemical, visual and computational investigation of this vascular component. The glycocalyx modulates leukocyte–endothelial interactions, thrombus formation and other processes that lead to microcirculatory dysfunction and critical organ injury in sepsis. It also acts as a regulator of vascular permeability and contains mechanosensors as well as receptors for growth factors and anticoagulants. During the initial onset of sepsis, the glycocalyx is damaged and circulating levels of glycocalyx components, including syndecans, heparan sulfate and hyaluronic acid, can be measured and are reportedly useful as biomarkers for sepsis. Also, a new methodology using side-stream dark-field imaging is now clinically available for assessing the glycocalyx. Multiple factors including hypervolemia and hyperglycemia are toxic to the glycocalyx, and several agents have been proposed as therapeutic modalities, although no single treatment has been proven to be clinically effective. In this article, we review the derangement of the glycocalyx in sepsis. Despite the accumulated knowledge regarding the important roles of the glycocalyx, the relationship between derangement of the endothelial glycocalyx and severity of sepsis or disseminated intravascular

coagulation has not been adequately elucidated and further work is needed.

Keywords: endothelial surface layer; glycocalyx; microcirculation; organ dysfunction; sepsis.

Introduction

The vascular endothelial surface is covered by the glycocalyx/endothelial surface layer (ESL), a gel-like layer that exhibits important effects that are antithrombotic and anti-inflammatory, and regulates vascular permeability [1]. The term ‘glycocalyx’ means ‘sweet husk’ and was first introduced in 1963 to describe an extracellular polysaccharide coating on the cells [2]. However, this structure has been challenging to study because it is extremely fragile and difficult to examine using ordinary light microscopy. As a result, extensive research on the glycocalyx has been relatively recent. The endothelial glycocalyx has three major components: membrane-binding proteoglycans (such as syndecan and glypican), glycosaminoglycan (GAG) side-chains conjugated with the core protein of the proteoglycans, and plasma proteins (such as albumin and antithrombin), as shown in Fig. 1 [3]. The multiple components of the glycocalyx are critical to its physiological function. The glycocalyx is synthesized by vascular endothelial cells and expressed on the endothelial cell surface [4]. The glycosaminoglycan structures of the glycocalyx can penetrate the intercellular clefts and can become contiguous with the basement membrane, but the term ‘glycocalyx’ is typically reserved for the structures lining the vascular lumen and is distinct from the basal extracellular matrix. It is also known that the similar structure expressed in the spaces between the basement membrane functions as a foothold for cells [5]. The glycocalyx on the luminal surface of the endothelial cells contributes to antithrombogenicity and fluid blood flow.

Following acute injury and inflammatory conditions, glucuronidases, including heparanases, reactive oxygen species (ROS) and other proteases, cause disruption of the glycocalyx and represent one of the earliest and most significant sites of injury during sepsis. After the shedding of

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Received: 8 June 2018

Manuscript handled by: P. H. Reitsma

Final decision: P. H. Reitsma, 28 September 2018

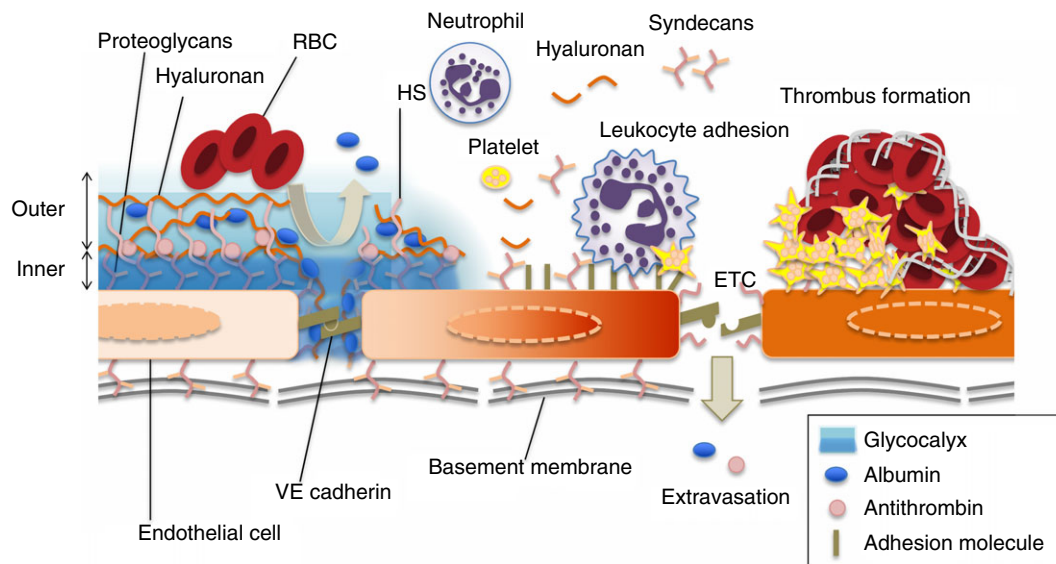


Fig. 1. Vascular endothelial damage and the glycocalyx. The glycocalyx has a two-layer structure. The inner layer is composed of a membrane-binding domain containing proteoglycan (e.g., syndecan) and conjugated oligosaccharides. The outer thicker layer is composed mainly of hyaluronan and plasma proteins (e.g. albumin). Antithrombin binds to its specific binding site, heparan sulphate. The glycocalyx contributes to various vascular functions such as antithrombogenicity, the suppression of cellular adhesion and selective permeability. The glycocalyx is easily destroyed, beginning at an early stage during sepsis, allowing leukocyte adhesion and thrombus formation. RBC, red blood cell; HS, heparan sulphate; ETC, endothelial cleft.

this structure, adhesion molecules such as E-selectin and intercellular adhesion molecule 1 are exposed on the denuded endothelium and induce the recruitment of leukocytes and platelets, leading to thrombus formation, which together with the massive fibrin formation results in circulatory dysfunction [6]. In addition, the destruction of the glycocalyx leads to capillary leakage, edema, accelerated inflammation, platelet aggregation, hypercoagulation and a loss of vascular responsiveness [4]. Subsequently, the altered blood flow and the impaired oxygen delivery result in organ failure. Although global oxygen delivery is typically increased during sepsis, many tissue capillary beds do not receive an adequate oxygen supply because of microvascular endothelial injury [7]. For example, pulmonary endothelial glycocalyx degradation increased the availability of endothelial surface adhesion molecules to circulating microspheres and contributed to neutrophil adhesion, which leads to diffuse alveolar damage, interstitial lung edema and clot formation during acute lung injury in sepsis [8]. The aim of this review is to summarize current knowledge regarding the glycocalyx and its derangement during sepsis. We describe methods used to measure the extent of injury and potential clinical applications. We also discuss potential therapeutic approaches to protect and restore this important vascular component.

Structure and function

Structure of the glycocalyx

The glycocalyx is a fragile structure covering the vascular endothelial surface. Its morphology and size vary

significantly depending on the method used for observation [4]. Even among the reports using transmission electron microscopy, there is significant variation depending on the fixation method used [9]. There have been many attempts to visualize the endothelial glycocalyx using electron microscopy and substitution of the original ruthenium-red staining with lanthanum or Alcian blue. In the review by Curry *et al.* [10], the thickness of the glycocalyx was reported to be 500 nm by the conventional Alcian blue labeling, 400–500 nm by microscopy and fluorescently-labeled dextran, and several micrometers by transmission electron with rapid freezing. Thus, further discussion is needed regarding standardizing the measurement of the glycocalyx thickness. Curry *et al.* [10,11] also reported that the endothelial glycocalyx consisted of a two-layered structure: an inner dense matrix layer associated with membrane-attached glycoproteins that forms a primary selective barrier to plasma macromolecules, and an outer less-dense layer composed mainly of GAGs and plasma proteins that extends one or more micrometers into the vessel's lumen and supports continuous blood cell movement while restricting inflammatory cell access to the endothelial surface (Fig. 1). This two-layer model hypothesis, however, remains to be proven. The glycocalyx is susceptible to various insults and is easily removed. During sepsis, for example, matrix metalloproteinases shed syndecan [12], and a disintegrin and metalloproteinase 15 (ADAM-15) cleaves CD44 [13]. Angiopoietin-2-induced heparanase mediates the degradation of heparan sulfate [14]. Hyaluronidase, thrombin,

elastase, plasminogen and cathepsin B destroy hyaluronan on the endothelial surface [12].

Although intravital microscopy has made *in vivo* evaluations possible, it does not allow direct visualization of the glycocalyx. The measured thickness is calculated by the distance between the erythrocytes and the endothelium (Figs. 2 and 3). Other methods for calculating glycocalyx thickness involve more sophisticated observation techniques, including confocal laser scanning microscopy [15], two-photon laser scanning microscopy [16] and atomic force microscopy [17]. Nevertheless, the thickness of the glycocalyx appears to vary substantially depending on the location, condition, observation techniques and vessel type, ranging from several nanometers to several micrometers. For instance, because the synthesis of the glycocalyx depends on the shear stress, it is thicker on the endothelial cells in arterioles than on those in venules (Fig. 2). Other than shear stress, endothelial cells are also exposed to pulsatile stretch and physical pressure that influences expression of the glycocalyx [18]. Immunofluorescent staining has revealed that cultured endothelial cells present reduced amounts of syndecan-1 (Fig. 4A). The expression of other glycosaminoglycans (such as heparan sulfate and chondroitin sulfate) is also diminished on the surfaces of cultured endothelial cells [9]. Although comparatively little glycocalyx is presented on the cellular surface, it is detected in the endothelial clef (i.e. the space between two contiguous endothelial cells) (Fig. 1). Thus, when the confluent endothelium is injured, and the gap expands, the syndecan-1 between the cells can be visualized (Fig. 4B). Considering these results, estimating the status of the glycocalyx under physiological conditions based solely on the results of *in vitro* studies seems difficult. This must be kept in mind when extrapolating experimental results to the physiological or clinical setting.

Regulation of cell adhesion

The recruitment of leukocytes to areas of infection is a precise multistep process involving tethering, rolling, adhesion and transmigration, which are all critical for efficient host defense. One of the most important physiological roles of the glycocalyx is vascular protection via the inhibition of leucocyte adhesion [4]. For example, Ishiguro *et al.* [19] reported that syndecan-4-null mice exhibit circulatory shock as a result of endotoxin and easily succumb to endotoxin injection. In the absence of infection, leukocyte-endothelial interactions are limited, occurring primarily in specialized vascular beds. Cell adhesion molecules on the endothelium, such as integrins and immunoglobulin super-families, are either hindered by or hidden within the glycocalyx. However, once infection occurs, the glycocalyx is targeted and shed by inflammatory mediators, such as histones and proteases. Along with the unveiling of the glycocalyx, adhesion molecules are exposed and facilitate ligand-receptor interactions

that promote the adhesion of leukocytes [20] (Fig. 1). In contrast, preservation of the glycocalyx mitigates leukocyte adhesion and subsequent vascular damage [21].

Maintenance of anticoagulation

The maintenance of its antithrombotic property is a key role of the glycocalyx. The intact endothelium has multiple anticoagulant properties, including the production and release of nitric oxide (NO), prostacyclin and tissue factor pathway inhibitor (TFPI) [22]. Endothelial cells also secrete heparan sulfate, which augments the anticoagulant action of antithrombin. Reportedly, circulating antithrombin in plasma binds to heparan sulfate located on the luminal surface and in the basement membrane of the endothelium [23]. Thrombomodulin is another endothelium-bound protein with anticoagulant and anti-inflammatory functions that are triggered in response to systemic procoagulant stimuli. Together with these critical anticoagulation functions, the glycocalyx plays an essential role in maintaining blood flow in the microcirculation. However, under septic conditions, disruption of the glycocalyx occurs and numerous inflammatory cytokines and mediators remove the protective effects of the endothelial surface, allowing leukocyte and platelet adhesion and microthrombi development [24]. These pathophysiologic responses function to isolate the infection and prevent the dissemination of microorganisms and are essential for the successful localization and elimination of microorganisms [25]. However, with overwhelming infection, these processes produce acute injury to the host and contribute to the overall morbidity and mortality of sepsis [26]. Typically, the excessive activation of coagulation leads to disseminated intravascular coagulation (DIC), and the disruption of the glycocalyx contributes to this status. Ikeda *et al.* [27] reported that syndecan-1 levels were associated with not only the severity of sepsis but also the development of DIC.

Regulation of vascular permeability

Vascular permeability was classically thought to be regulated according to the traditional Starling principle, where filtration across the microvasculature was simply determined by the opposing hydrostatic and oncotic forces within the vascular lumen and interstitial space. However, the revised Starling equation expands upon this concept, in part by identifying the glycocalyx as the structural determinant of the oncotic gradient [28]. Current understanding of the factors that maintain vascular permeability, in addition to the glycocalyx, includes other important junctional proteins, adhesion molecules represented by VE-cadherin, and vascular endothelial protein tyrosine phosphatase cytoskeletons that are important for this complex regulation [29]. Endothelial glycocalyx injury and increased capillary permeability leading to tissue edema,

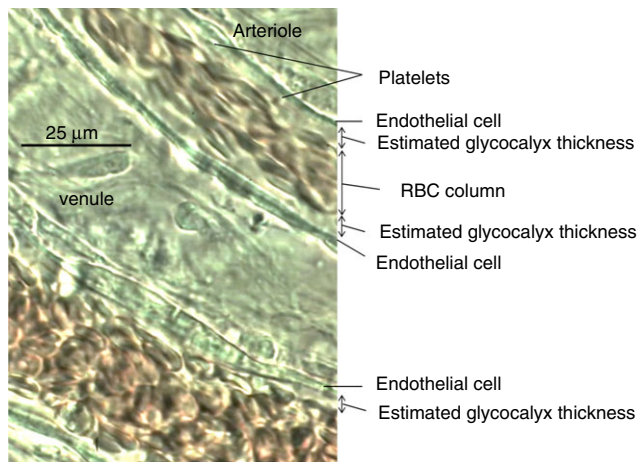


Fig. 2. Intravital microscopic view of an arteriole and venule recorded using a high-speed camera. The glycocalyx is visible as the space between the endothelium and the red blood cell column. Because the endothelium in the arteriole is exposed to a higher shear stress than that in the venule (as can be recognized based on the different shapes of the red blood cells), the glycocalyx is thicker in arterioles than in venules. The images represent the healthy microcirculation captured in the rat mesenteric arteriole and venule. RBC, red blood cell.

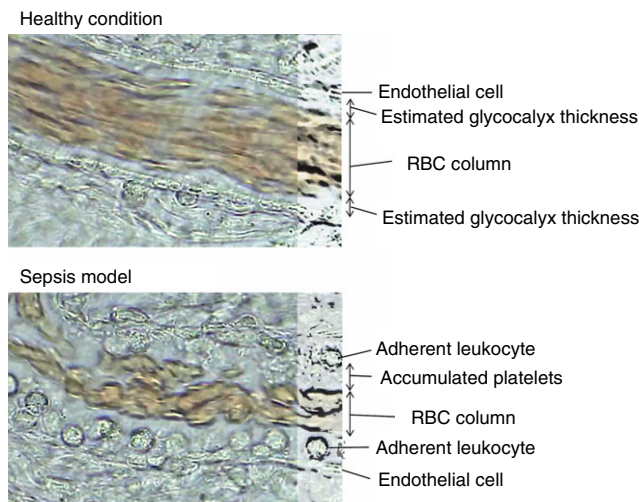


Fig. 3. Changes in the microcirculation after endotoxin administration. Smooth circulation is observed under normal conditions (top). Endothelial damage as expressed by the thickness and roughness of the endothelial cells, the adhesion of the round and stiff leukocytes, and platelet piling on the endothelium, together decrease the blood flow. The glycocalyx was thought to be disrupted in this vessel (bottom).

suggest the glycocalyx acts as a competent barrier against the passage of water and colloids. The glycocalyx regulates the fluid shift and maintains fluid volume, acting as a negatively charged sieve. There are five types of GAG: up to 90% is heparan sulfate, with the remainder consisting of hyaluronic acid, chondroitin sulfate, dermatan sulfate and keratin sulfate. Among them, hyaluronic acid does not

bind to a core protein and forms a viscous solution with water because it is highly hydrophilic [30]. The sulfated glycosaminoglycans sequester water similar to chondroitin sulfate in aggrecan in cartilage. GAGs carry a significant number of negatively charged binding sites depending on the sulfation, and changes in sulfation affect the protein binding and vascular permeability [4]. Under physiologic conditions, plasma proteins are bound within the glycocalyx [31].

The mechanism responsible for the increased vascular permeability as a result of glycocalyx degradation remains to be established. There are two pathways for the leakage of water and macromolecules: a transendothelial pathway and a paracellular pathway [32]. The endothelial cleft regulates the paracellular pathway, and proinflammatory factors such as ROS and tumor necrosis factor- α (TNF α) stimulate the opening of tight junctions, adherent junctions and gap junctions during sepsis [28]. Proinflammatory mediators are also known to accelerate caveolae formation, leading to transendothelial hyperpermeability during sepsis. Similarly, atrial natriuretic peptide stimulates caveolae-mediated transendothelial albumin transport [33]. Both the transcellular and paracellular transport of water and macromolecules during sepsis result in increased interstitial edema and a decrease in plasma proteins.

Receptor for signals

The glycocalyx acts as a mechanoreceptor on endothelial cells and senses the shear stress induced by viscous blood flow [34]. An increased fluid shear increases NO production, which in turn dilates vessels and reduces the adherence of leukocytes and platelets [35]. Higher shear stress is also known to increase albumin uptake and alter glycocalyx properties (such as increased thickness) [15]. As previously described, endothelial cells cultured under static conditions express little glycocalyx, but expression increases dramatically when the cells are exposed to shear stress [36]. Damage to the glycocalyx impairs these mechanisms and perturbs the endothelial response to mechanical stresses.

Besides physical stress, the binding of ligands and enzymes to the glycocalyx also induces signal transduction and enzymatic modification. The activities of growth factors, such as fibroblast growth factor and epithelial growth factor, depend on interactions with the glycocalyx [37,38]. Antithrombin (also known as heparin cofactor I), a serine protease inhibitor, is well known to bind to heparan sulfate proteoglycans (syndecan-4), which increases its inhibitory activity [39]. Kaneider *et al.* [40] investigated the effect of antithrombin on the endotoxin-induced adhesion of neutrophils to endothelial cells *in vitro* and reported that leukocyte adhesion to the endothelium is reversed by the ligation of antithrombin with syndecan-4. The other serpin, heparin cofactor II, is also activated by binding to dermatan sulfate [41]. TFPI binds to inhibit

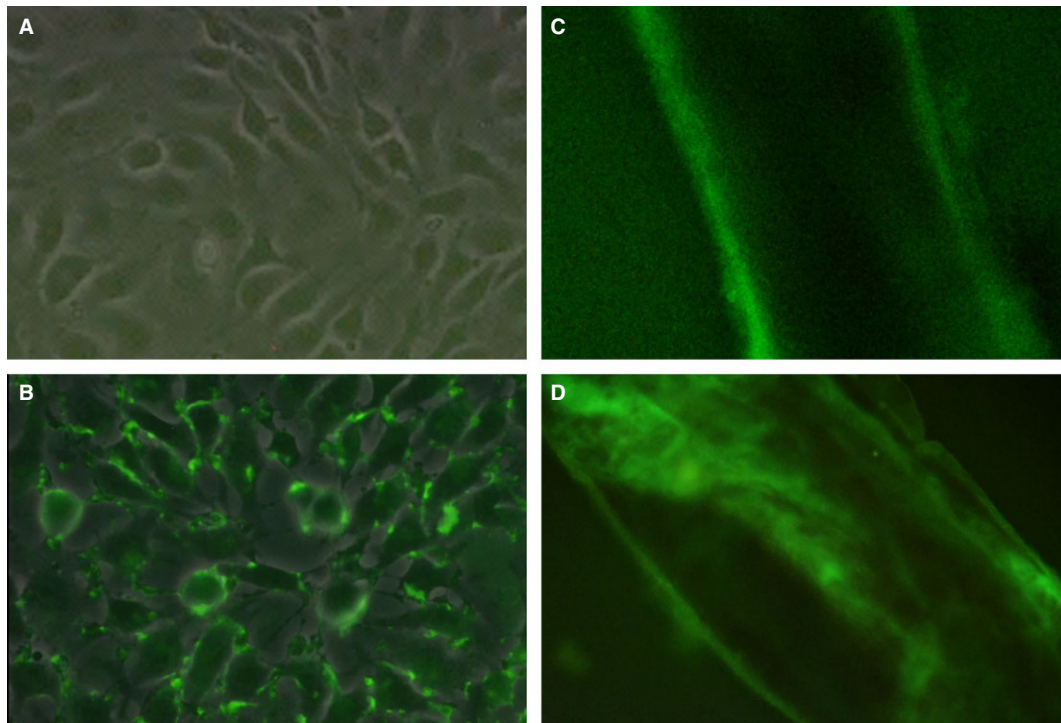


Fig. 4. Cultured rat aortic endothelial cells and rat mesenteric vascular endothelium with immunofluorescent staining for syndecan-1. The cultured endothelial cells show almost no very fine immunofluorescence under the static condition (A), whereas immunofluorescence in the endothelial clefts is visible in the area surrounding the damaged endothelial cells (the endothelial damage was induced by $50 \mu\text{g mL}^{-1}$ of histone H4; syndecan-4 was stained with immunofluorescence and the image was taken 8 h after the exposure to histone) (B). The vascular endothelium is visible as a smooth, homogenous lining of syndecan-1 on its surface under normal condition (C). This syndecan-1 lining became thinner and was disrupted after endotoxin treatment (D). The green fluorescence depicted the staining for syndecan-1.

factors VIIa and Xa via heparin sulfate [42]. The glycocalyx also has the ability to bind enzymes that scavenge ROS, such as extracellular superoxide dismutase. These enzymes help to reduce oxidative stress and maintain NO bioavailability [3]. Taken together, these findings suggest that the glycocalyx acts as a receptor for physical and chemical signals, inducing the physiological responses of the vascular endothelium.

Evaluation of glycocalyx damage

Measurement of glycocalyx in plasma and urine

To evaluate the derangement of endothelium clinically, some investigators have measured transmembrane proteins such as thrombomodulin, adhesion molecules and receptors. Others have measured extracellular vesicles or circulating endothelial progenitor cells. Because the endothelial cell is multi-potential, there is no one-size-fits-all biomarker. The measurement of circulating glycocalyx components is one way to evaluate endothelial function. Shedding of the glycocalyx is promoted by various inflammatory mediators, including ROS, $\text{TNF}\alpha$, heparanase and matrix metalloproteases, during sepsis [43]. In addition, hyaluronidase and serine proteases, such as

thrombin, elastase, proteinase 3, plasminogen and cathepsin B, are also involved in the derangement of the glycocalyx [12]. The levels of various components of the glycocalyx (e.g. syndecan-1, endocan and heparan sulfate) have been proposed as markers of glycocalyx degradation [44]. The reliability of circulating glycocalyx components as endothelial damage biomarkers has not been widely accepted because of the paucity of evidence in support of this concept. To date, several experimental and clinical studies have reported that the degree of glycocalyx shedding correlates with the severity of various illnesses, such as cardiovascular diseases, kidney diseases and trauma [45–47]. Similarly, the usefulness of measurements of glycocalyx components has also been introduced in the treatment of sepsis and septic shock [30,48–50]. However, there is insufficient direct evidence demonstrating that circulating levels of glycocalyx components correlate with endothelial injury. Further work needs to be performed to better understand what useful biomarkers can be evaluated for glycocalyx shedding.

Regarding syndecan, four subtypes are known to exist. The distribution of each subtype of syndecan is unique, and syndecan-1, -2 and -4 are expressed between vascular endothelial cells and on the side of vascular endothelial cells closer to the basal membrane [12]. Nelson *et al.* [51]

measured all subtypes of syndecan and reported increased syndecan-1 and syndecan-3 levels in critical conditions, whereas no elevation in the plasma levels of syndecan-2 or syndecan-4 was detected. Ostrowski *et al.* [52] conducted syndecan-1 measurements and thromboelastography simultaneously during sepsis and reported that syndecan-1 shedding is associated with hypocoagulability based on prolonged R-times on thromboelastography, presumably representing the release of heparan sulfate fragments (> 5 saccharides in size) into the circulation. As a result, although the denuded endothelial surface could be more prothrombotic, shedding heparan into the plasma itself is antithrombotic. Syndecan-4 is known as a binding site of antithrombin, and its derangement decreases the activity of antithrombin [47]. Strand *et al.* [53] reported that the shedding of syndecan-4 promotes leukocyte recruitment after a lipopolysaccharide challenge in mice.

Other fragments of the glycocalyx that are released into the plasma, such as hyaluronic acid and heparan sulfate, have been used as biomarkers of endothelial injury [49]. Besides its usefulness as a biomarker, circulating heparan sulfate has also been reported to reduce inflammation by binding and inhibiting the downstream signaling of circulating inflammatory mediators, including histones and high mobility group protein B1 (HMGB1), during sepsis. Soluble heparan sulfate released during endothelial glycocalyx degradation may intercept histones and HMGB1, preventing their binding to the endothelial cell surface [54]. The protective effect of glycocalyx fragments is complex and multifactorial. Studies note that heparan sulfate fragments stimulate the release of proinflammatory cytokines [55] or low-molecular-weight hyaluronan [56], which can act on TLR-4 receptors to increase inflammation. The complexity of glycosaminoglycan signaling requires further study.

Antithrombogenic activity assay

Dimitrievska *et al.* [57] developed an *in vitro* assay that can quantify the functional heparin weight on live endothelialized surfaces and the anticoagulant capacity to inactivate factor Xa and thrombin. They reported an approximately 10-fold difference in heparin weight between native aorta and cultured human umbilical vein endothelial cells (HUVECs), with HUVECs demonstrating a 5-fold lower anticoagulant capacity in inactivating both factor Xa and thrombin relative to native aorta tissue. Because a method to characterize glycocalyx functionality has not yet been developed, this method might be valuable in future studies of endothelial anticoagulation activity.

Evaluations using electron microscopy and intravital microscopy

Intravital microscopy and electron microscopy have been used to visualize the glycocalyx. Okada *et al.* [58]

visualized different ultrastructural characteristics of the endothelial glycocalyx in continuous (lacking fenestrations), fenestrated (e.g. renal gastrointestinal capillaries) and sinusoidal (e.g. hepatic sinusoids) capillaries. The glycocalyx layer contained both continuous and fenestrated capillaries that were thicker than in the sinusoids. In the kidney, the glycocalyx began to occlude the endothelial pores of the fenestrated capillaries. In the hepatic sinusoids, the glycocalyx covered the luminal side and the side facing the space of Disse. They also showed disrupted capillary endothelial glycocalyx, such as peeling away from the cells and clumping, in an animal model of sepsis [59].

Intravital microscopy is another technique used to observe the vessels in living animals at a high resolution. Kataoka *et al.* [60] reported that the thickness of the glycocalyx in healthy mice was $1.07 \pm 0.39 \mu\text{m}$ and that it decreased to $0.36 \pm 0.15 \mu\text{m}$ in septic mice. Although intravital microscopy by itself can only be used for indirect observations, direct observations become possible when used together with immunofluorescent staining (Fig. 4C, D). By intravital microscopy, the glycocalyx is recognized as a transparent zone between the endothelium and the red blood cell (RBC) column (Figs. 2 and 3). Because the glycocalyx repels RBCs because of its electric charge, it produces an exclusion zone running parallel to the endothelium and adjoining the central column of the RBCs. Prior work by Smith *et al.* [61] reported that high-resolution near-wall fluorescent microparticle image velocimetry enables measurement of the velocity profiles in the red cell-depleted plasma layer near the endothelial lining. The radial positions of fluorescently labeled microspheres in an optical section through the midsagittal plane of each vessel can be used to determine fluid particle translational speeds. Although intravital microscopic examination using conventional light microscopy is not readily performed in clinical settings, side-stream dark-field (SDF) microscopy has been reported and offers a novel diagnostic tool that has the potential to be further developed for clinicians. Two-photon laser scanning microscopy is another novel technique that can directly visualize larger vessels, providing a detailed image of the endothelial surface and enabling the identification of the glycocalyx [62]. Using this technique, the quantification of fluorescent-labeled leukocytes in the vasculature and a fluorescence-labeled dextran permeability analysis become possible.

Evaluation using side-stream dark-field imaging

Most microvascular disturbances elicit changes in the glycocalyx that are reflected by the increased penetration of RBCs into the glycocalyx (Fig. 5, left). *In vivo* video microscopy technologies allow the direct assessment of the endothelial glycocalyx by measuring the extent of penetration of RBCs into the endothelial glycocalyx [63]. Side-stream dark-field

(SDF) imaging visualizes hemoglobin in RBCs within the organ microvasculature using a reflected light-emitting diode (LED) light [64]. Under SDF imaging, the RBCs in the microvasculature maintain a certain distance from the surface of the endothelium, and the thickness of the glycocalyx can be calculated mathematically in living animals, including humans, without causing harm [65]. In addition, recent advances in computer technology have made it possible to measure the thickness of the glycocalyx automatically by computation means (Glycocheck BV, Maastricht, the Netherlands) [66–68]. Under septic conditions, a perturbed glycocalyx allows the erythrocytes to penetrate deeper towards the endothelium, resulting in an increase in the perfused boundary region (PBR) (Fig. 5, right). The PBR represents the permeable part of the endothelial glycocalyx that allows flowing RBCs to move toward the endothelial surface, which increases in the PBR, reflecting a decreased thickness of the glycocalyx [69].

Lee *et al.* [66] examined the sublingual microcirculation in over 900 participants in a population-based, prospective cohort study and reported a strong association between changes in the endothelial glycocalyx properties calculated as the PBR and microvascular perfusion. However, it is also known that PBR measurements do not always translate to glycocalyx thickness. The mechanical calculation is set to measure the perturbation of RBCs in microvessels within a mean inner lumen between 5 and 25 μm . Because it is not likely that smaller or larger vessels have the same glycocalyx constitution and the thickness of the less-dense outer layer and that of the denser inner layer will be different depending on the vessel size, the measurement result may not be comparable between the different sizes of the vessels. Furthermore, because the measurement is also affected by the blood flow, the calculation should be modified by the flow speed [28,31–34]. Together with the PBR, the vascular density and the RBC filling percentage are calculated independently. However, few studies have examined these parameters during sepsis [65]. We measured the changes in PBR, vascular density and RBC filling percentage in a lipopolysaccharide-induced rat model of sepsis. As a result, changes in the PBR of the intestinal wall microvasculature were recognized as early as 30 min after endotoxin infusion. Interestingly, mirror-image changes were recognized in the RBC filling percentage. The density of the perfused vessel starts to decrease at 2 h and then continuously decreases over time (Fig. 5, right).

Therapeutic approaches for protecting the glycocalyx

Fluid management

Fluid management has an important influence on the integrity of the glycocalyx. Hypervolemia is injurious to the glycocalyx and glycocalyx damage can be limited by avoiding hypervolemia. The rapid administration of crystalloid or colloid fluids accelerates endothelial glycocalyx shedding

[70,71], and proposed mechanisms include hemodilution of the plasma components and potential osmotic changes [71]. Hypervolemia itself damages the glycocalyx, but it also triggers the release of atrial natriuretic peptide that denudes the endothelial surface layer and causes rapid shedding of components of the glycocalyx. Bruegger *et al.* [72] demonstrated that infusion of atrial natriuretic peptide induced an increase in vascular permeability, a histological degradation of the glycocalyx and an increased release of syndecan-1 of up to 18-fold in an isolated heart model. They also reported an increase in plasma atrial natriuretic peptide has been found to precede shedding of the glycocalyx in patients undergoing coronary bypass surgery. Some reports have shown a preferable effect of hydroxyethyl starch (HES) [73] compared with crystalloids [71,74], whereas others have reported that the effect of albumin was superior to HES [75,76] for volume repletion. The vascular glycocalyx also serves as an effective buffer barrier for sodium, and hyponatremia damages the glycocalyx, removing the barrier function that facilitates sodium entry into endothelial cells [77].

Glycemic control

Hyperglycemia has been known to degrade the glycocalyx. The thickness of the glycocalyx in diabetic patients is reported to be half of that in healthy controls, and patients have higher plasma levels of hyaluronan [78]. ROS and the activation of glycocalyx-degrading enzymes are suspected causes of the damage. Importantly, the damage occurs not only in chronic hyperglycemia but also in acute hyperglycemic conditions [79]. Thus, glycemic control and insulin therapy for sepsis patients with hyperglycemia might be useful for protecting the glycocalyx.

Corticosteroids

The protective effect of corticosteroids in sepsis is uncertain. Some studies have reported that glucocorticoids limit inflammatory glycocalyx injury by suppressing cytokine and chemokine production and by preventing inflammatory cell migration [80]. Reportedly, hydrocortisone also significantly reduced the shedding of the endothelial surface layer after ischemia/reperfusion in an *ex vivo* model [81]. A similar effect was also reported in a $\text{TNF}\alpha$ -induced heart perfusion model [82]. However, the efficacy of corticosteroids in protecting the glycocalyx in patients with sepsis has not yet been confirmed.

Plasma proteins

Some plasma proteins may protect the glycocalyx [83]. The outer layer of the glycocalyx contains the non-sulfated, non-negatively charged, high-molecular-weight polysaccharide hyaluronan as a dominant component, and albumin, in particular, binds to this component [11]. Glycocalyx-bound albumin plays a key role in stabilizing and protecting the

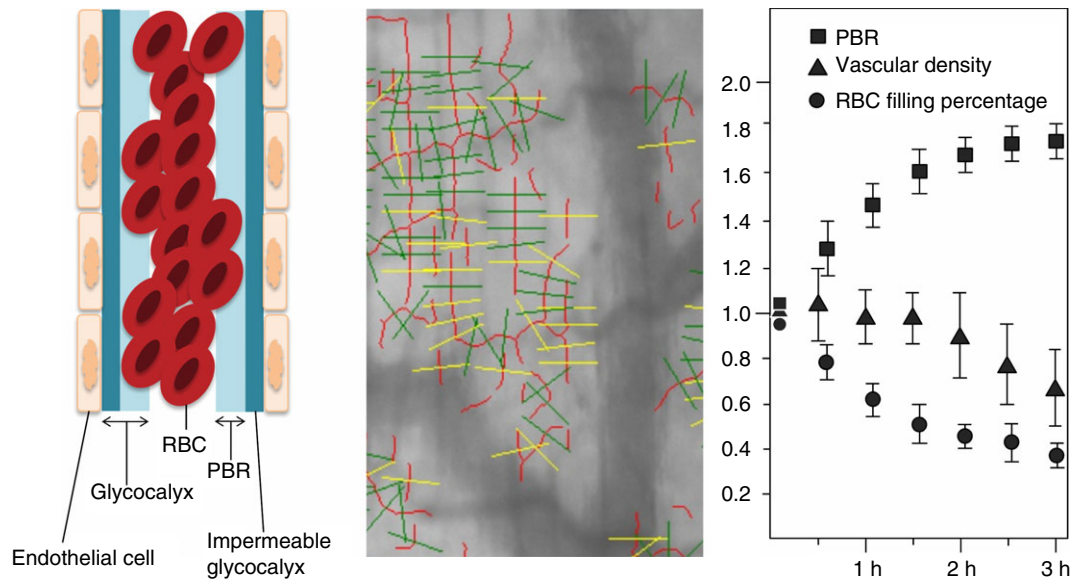


Fig. 5. Observation of the glycocalyx using the side-stream dark-field imaging technique in a rat model of sepsis. Left: depiction of the concept of glycocalyx thickness as suggested by lateral red blood cell (RBC) movement. The dynamic lateral red blood cell movement into the glycocalyx is expressed as the perfused boundary region (PBR). Middle: the software places vascular segments every 10 μm along the length of the vessels. Subsequently, a sequence of 40 frames is recorded containing 300 major vascular segments. The observer moves the camera to a different location until a minimum total of 3000 vascular segments are placed. The software automatically discriminates a valid vascular (green lines) segment from the invalid segment (yellow lines). Then, the valid vascular segments are visualized as the valid microvasculature (red lines). The experiment was performed using a lipopolysaccharide-induced sepsis rat model; 8 mg kg^{-1} of lipopolysaccharide was injected from the tail vein and the intestinal microcirculation was observed from time 0 to 3 h. Right: changes in PBR, RBC filling percentage and valid vascular density after endotoxin infusion. The PBR began to increase at 30 min after the endotoxin infusion and reached 1.7-fold of the baseline value. The RBC filling percentage started to decrease at the same time as that for the PBR. The density of the perfused vessel started to decrease at 2 h.

glycocalyx and bound albumin to form an interconnected structure on the endothelial surface. This gel-like outer layer of the glycocalyx is stabilized by the interaction of its components to facilitate proper function. Hyaluronan-bound albumin reduces the hydraulic conductivity of the vascular barrier and helps to retain albumin through its negative electric charge [84]. The sulfation patterns of the inner glycocalyx also have a negative charge, and the net negative charge contributes to the repulsion of negatively charged molecules, including albumin, leukocytes, RBCs and platelets [53]. The bound albumin is also expected to act as a histone scavenger on the endothelium [85]. Consequently, the loss of circulating albumin accompanies the loss of the glycocalyx, increasing fluid extravasation [86]. In animal models, albumin decreased the net fluid extravasation, compared with HES or crystalloids [86].

Another plasma protein, antithrombin, acts as the predominant thrombin inhibitor on the vascular surface through its binding to heparan sulfate side-chains of the glycocalyx [87]. Heparan sulfate contributes to the antithrombotic capacity of the endothelium, and constitutes 60–90% of the total amount of the glycocalyx and is presumably the most important functional GAG [3]. Antithrombin's anticoagulant ability is also known to increase dramatically when it binds to heparans. Antithrombin interactions with the glycocalyx augment its protease inhibitor function, which

decreases inflammatory responses and contributes to the stabilization of the glycocalyx [57]. As previously described, antithrombin is considered to confer its critical activity after binding to heparan sulfate on the endothelial vascular surface [40]. Chappell *et al.* [88] reported that antithrombin could reduce the shedding of the endothelial glycocalyx in a heart perfusion model of ischemia/reperfusion. Antithrombin's antithrombotic and anti-inflammatory functions are thought to be expressed through the protection of the glycocalyx [89]. Although more than 90% of the circulating antithrombin is composed of α -antithrombin, β -antithrombin has a higher affinity to heparan sulphate and has been shown to protect endothelial cells more efficiently than the α -isoform [90]. Potentially, a β -isoform-rich antithrombin formulation might be more efficient than currently available antithrombin concentrates. The efficacy of the other physiological anticoagulants such as thrombomodulin and tissue factor pathway inhibitor can be assumed; however, the evidence is still lacking.

Heparin and heparinoids

Low-molecular-weight heparin (LMWH) is expected to suppress structural changes in the glycocalyx as a result of inflammation. In an animal model, the stabilization and prevention of glycocalyx shedding by LMWH

following heparanase inhibition was reported [91]. Similarly, a protective effect of unfractionated heparin was shown in an animal model of sepsis [92]. However, heparins are also known to interact with heparan sulfate and to release heparan sulfate from the glycocalyx competitively [93]. Because those effects in animal models have not been confirmed clinically, further investigation is required.

Sulodexide, a heparin sulfate-like compound that is resistant to degradation by heparanase, reportedly accelerates endothelial glycocalyx regeneration. This effect has been demonstrated both *in vitro* and *in vivo*. In the latter case, reduced vascular permeability was associated with improved animal survival, even when the animals were treated 2 h after the induction of sepsis [94].

Glycocalyx components

The potential efficacy of the administration of exogenous hyaluronan in restoring the glycocalyx has been reported [95]. Although ROS production is known to be upregulated during sepsis and is the major contributor to hyaluronan degradation, the efficacy of an exogenous superoxide dismutase-catalase was expected [92]. Although complicated, hyaluronan degradation products are known to induce ROS production and the antioxidant N-acetyl-l-cysteine to protect glycocalyx shedding [96].

Heparan sulfate is expected to restore the glycocalyx when it is shed from endothelial cells. Artificial heparan sulfate restoration using exogenous heparan sulfate with or without the glycocalyx regenerator and protector sphingosine 1-phosphate has been reported [97]. The effect of supplemental therapy using components of the glycocalyx with the goal of restoration requires further investigation.

Conclusion

The role of the glycocalyx in maintaining microcirculatory function is important as a potential therapeutic target for sepsis. It is also necessary to consider the influence on the glycocalyx whenever we plan therapeutic strategies for sepsis, such as fluid resuscitation, and albumin and antithrombin administration. Although new scientific techniques have enabled further understanding and detection of endothelial glycocalyx derangement, these approaches have yet to guide management and therapeutic approaches in septic patients that improve outcomes. In septic patients, further work is needed to understand the relationships among the derangement of endothelial glycocalyx, the severity of sepsis and DIC, in order to develop therapeutic strategies based on the findings.

Acknowledgements

A part of this report was presented at the 62nd Annual Scientific and Standardization Committee meeting of

the International Society on Thrombosis and Haemostasis.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interests.

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