Role of the Glycocalyx as a Barrier to Leukocyte-Endothelium Adhesion

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Abstract

Leukocyte (WBC) to endothelial cell (EC) adhesion is a receptor-mediated process governed by the avidity and affinity of selectins, which modulate adhesive forces during WBC rolling, and integrins, which determine the strength of firm adhesion. Adhesion receptors on the EC surface lie below an endothelial surface layer (ESL) comprised of the EC glycocalyx and adsorbed proteins which, in vivo, have a thickness on the order 500 nm. The glycocalyx consists of a matrix of the glycosaminoglycans heparan sulfate and chondroitin sulfate, bound to proteoglycans and encased in hyaluronan. Together, these carbohydrates form a layer that varies in glycan content along the length of post-capillary venules where WBC-EC adhesion occurs. Thickness and porosity of the glycocalyx can vary dramatically during the inflammatory response as observed by increased infiltration and diffusion of macromolecules within the layer following activation of the EC by cytokines and chemoattractants. In models of inflammation in the living animal, the shedding of glycans and diminished thickness of the glycocalyx rapidly occur to facilitate penetration by the WBCs and adhesion to the EC. The primary effectors of glycan shedding appear to be metalloproteases and heparanase released by the EC. Retardation of glycan shedding and WBC-EC adhesion has been demonstrated in vivo using MMP inhibitors and low-molecular-weight heparin (LMWH), where the latter competitively binds to heparanase liberated by the EC. Together, these agents may serve to stabilize the ESL and provide a useful strategy for treatment of inflammatory disorders.

1 Introduction

The inflammatory process revolves around a sequence of events that leads to emigration of leukocytes (WBCs) through the microvascular wall into the tissue space. Convective transport of WBCs to the microvasculature leads to their radial migration to the microvessel wall (margination), rolling along the endothelium and firm adhesion to the endothelium (EC) prior to diapedesis (Atherton and Born 1972, 1973; Grant 1973), as depicted in Fig. 1. As blood traverses the arteriolar network, hemodynamic

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B. M. Fu, N. T. Wright (eds.), *Molecular, Cellular, and Tissue Engineering of the Vascular System*, Advances in Experimental Medicine and Biology 1097,
https://doi.org/10.1007/978-3-319-96445-4_3

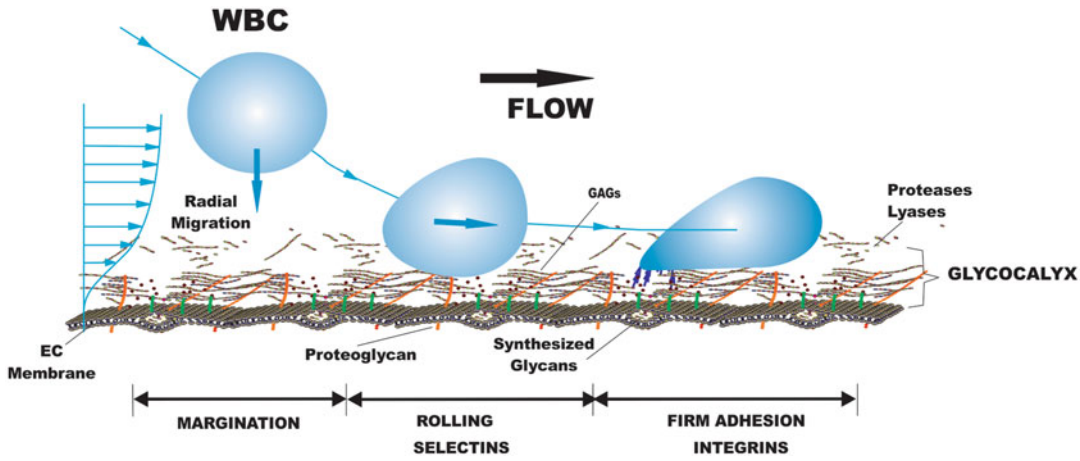

Fig. 1 Leukocyte-endothelium adhesion in post-capillary venules is an essential step in the inflammatory process. As WBCs exit the capillaries, hemodynamic forces and interactions with red cells cause the radial migration of WBCs to the EC surface (margination) with subsequent rolling along and firm adhesion to the EC. WBC rolling is facilitated by the selectin family of adhesion molecules that maintains WBC contact with the EC surface. Arrest and firm adhesion follow due to the strong adhesion mediated by integrins on the WBC and their receptors on the EC. The selectins and integrin receptors are buried within the endothelial surface layer (ESL) that consists of the EC glycocalyx and adsorbed proteins. The ESL is typically about 500 nm thick and shields selectins and integrin receptors that protrude from 20 to 40 nm above the EC membrane

and topographical features promote WBC-EC interaction (Bagge and Karlsson 1980; Braide et al. 1984; Goldsmith and Spain 1984; Schmid-Schonbein et al. 1980). As blood exits from capillaries to post-capillary venules, WBC radial migration to the EC occurs due to hemodynamic forces and red blood cell (RBC) interactions (Schmid-Schonbein et al. 1980). Rolling of WBCs on the EC is then promoted by adhesive interactions with the molecular surface layer on the EC and receptor-mediated adhesion with the selectin family of carbohydrates (Springer and Lasky 1991). Subsequent firm adhesion of WBCs ensues due to receptor-mediated adhesion of integrins on the WBC surface to counter receptors on the EC (Zarbock and Ley 2009; Springer 1990). Successful completion of the adhesion process hinges on the availability of ligands in the EC surface layer (ESL) formed by the EC glycocalyx and adsorbed proteins (Pries et al. 2000; Reitsma et al. 2007; Weinbaum et al. 2007).

The interface between blood and endothelium has been of interest for decades in light of its role in inflammation, permeability to macromolecules, and thrombosis. Early studies on the structural makeup of the capillary wall drew attention to the surface of the endothelium as an essential part of the "hematoparenchymal barrier" (Zweifach 1955). The observations of microvascular function recognized that endothelial cells continuously secrete substances that form an "intercellular cement" and the basement membrane. With advances in intravital microscopy, direct visualization of the dynamics of blood-endothelial cell (EC) interactions in the microcirculation led to hypotheses to explain the basis for blood cell to EC adhesion, the clotting of blood, and the transvascular exchange of fluid and macromolecules. It is now recognized that the surface of the endothelium is coated with a layer of polysaccharides and transmembrane proteins, as described in Chap. 1, that was subsequently visualized by electron microscopy by Bennett and others (Bennett et al. 1959; Luft 1966). In view of its predominant polysaccharide constituents, Bennett (Bennett et al. 1959) termed it the "glycocalyx," as derived from the Latin for "sweet husk." Initially viewed as an extension of the endothelial cell basement membrane onto the luminal surface of the EC, the fine structure of the glycocalyx has been described as a network

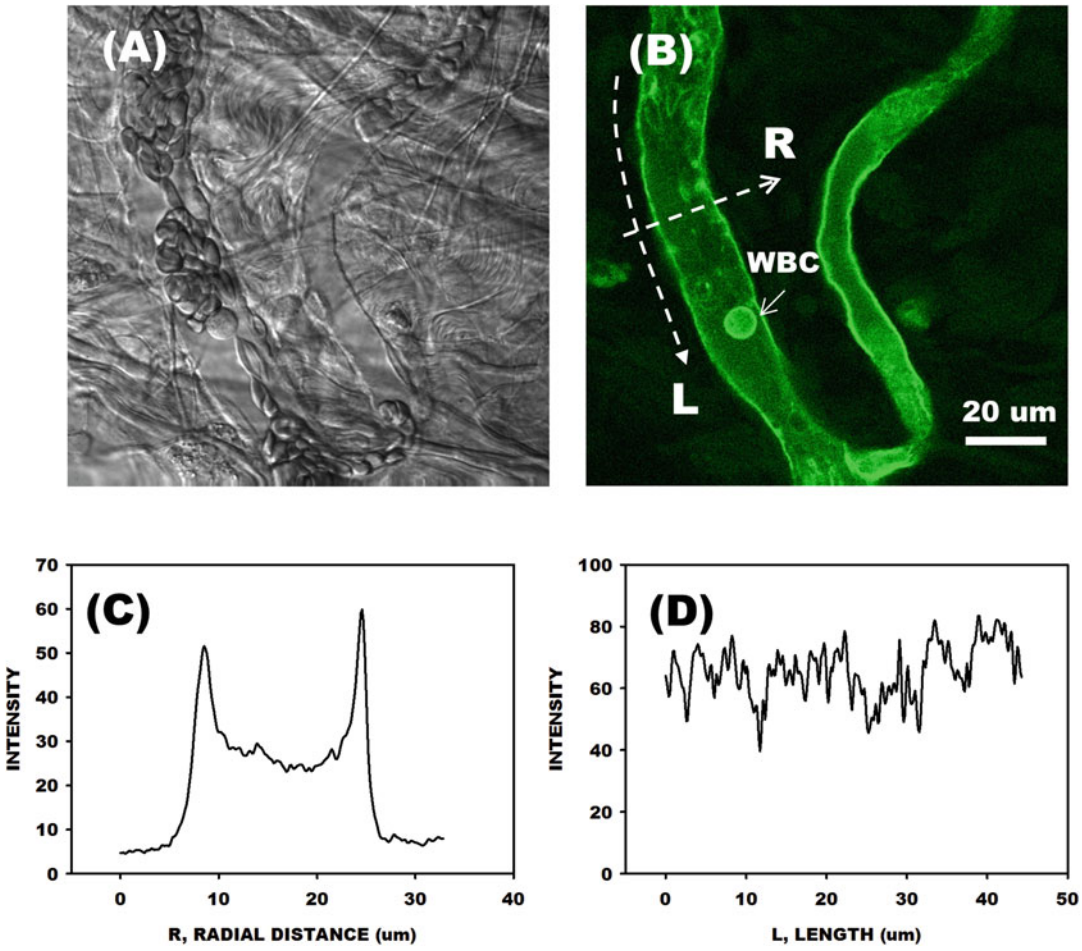

Fig. 2 Visualization of the endothelial glycocalyx. (**a**) Bright-field view of post-capillary venules in mesentery of the rat. (**b**) Fluorescence microscopy of the glycocalyx labeled with the fluorescently labeled lectin BS-1. (**c**) The average radial profile of fluorescence along the measurement line R shows a peak value at each wall with intensity proportional to the concentration of lectins bound to the EC surface. Reductions in peak fluorescence were taken as a measure of the shedding of glycans from the EC surface. (**d**) Variation of fluorescence intensity with length L along a wall varies ±50% as shown for the wall next to the dashed line

of glycoproteins on the order of 50–100 nm thick, with a characteristic spacing of 20 nm that accounts for the resistance to filtration of small molecules (Squire et al. 2001). Recognition that the EC surface contains an abundance of negatively charged carbohydrates (Simionescu et al. 1982) led to the use of lectins to visualize the endothelial surface layer (Schnitzer et al. 1990a). Visualization of the glycocalyx with lectin staining is illustrated in Fig. 2, where the surface of post-capillary venules is stained with the fluorescently labeled lectin BS-1 (*Bandeiraea simplicifolia*) (Mulivor and Lipowsky 2004). Lectins are carbohydrate-binding proteins that may be used to loosely identify specific glycoproteins in the EC surface layer (Schnitzer et al. 1990b). As shown in Fig. 2a, staining of the glycocalyx reaches a maximum at the vessel walls. The average radial profile of fluorescence along the length of a microvessel reveals a peak intensity that is proportional to concentration of lectin-binding sites on the EC surface. Peak staining along the length of one wall (Fig. 2d) illustrates the variability of glycans on the surface, which

may vary ±50% about the mean. As shown in the following, peak intensity of glycan staining may be used to quantify the shedding of glycans during the inflammatory process and changes in glycan concentration with hemodynamic (shear) conditions.

2 Microvascular Hemodynamics

Historically, the role of the glycocalyx in affecting microvascular hemodynamics arose from the seminal studies of Klitzman and Duling (1979) and Desjardin and Duling (1990) in their studies of the basis for the anomalous levels of capillary hematocrit observed in most tissues by intravital microscopy. At that time, studies subsequent to the pioneering observations of reduced small vessel hematocrit by Poiseuille (1835) and Fahraeus (1929) noted reductions in capillary hematocrit that were well below 50% of systemic values (Pries et al. 1990; House and Lipowsky 1987a). Average values of capillary hematocrit on the order of 10–20% of systemic hematocrit far exceeded the hypothetical maximum reduction of 50%, based upon red cell velocity profiles in small tubes (Sutera et al. 1970) where, for a parabolic velocity profile, peak velocity along the vessel center line may reach a maximum of twice the mean velocity. Klitzman and Duling (1979) hypothesized that the low capillary hematocrits arose from retardation of fluid on the endothelial cell surface. To validate this hypothesis and explore the role of the glycocalyx in contributing to the anomalous low capillary hematocrits, Desjardin and Duling (1990) inserted finely drawn micropipettes into feeding vessels and perfused individual capillaries with heparinase to strip off the glycocalyx. Their results showed a twofold rise in capillary hematocrit, presumably due to the resultant increase in the effective capillary diameter with degradation of the glycocalyx. Subsequent studies have shown increases in capillary hematocrit in response to its removal by perfusion with hyaluronidase (Cabrales et al. 2007) or degradation due to the presence of reactive oxygen species derived from oxidized LDL (Constantinescu et al. 2001).

To delineate the hemodynamic significance of the glycocalyx insofar as it affects the resistance to blood flow, studies have explored the effects of its enzymatic removal by direct intravital microscopy. Measurements by Pries et al. of regional pressure drops and flows in the mesenteric microvasculature following enzymatic removal of the glycocalyx, by perfusion with heparinase, suggested a 14–20% decrease in the resistance to flow (Pries et al. 1997). Their analysis of this diminished resistance suggested that removal of the glycocalyx theoretically increased microvessel diameter throughout the network by about 1 μm. Consistent with these findings, a hydrodynamically significant glycocalyx has been explicitly shown by analysis of the velocity profiles of small fluorescent microspheres in the in vivo microcirculation using techniques of particle image velocimetry (PIV) (Potter and Damiano 2008; Smith et al. 2003). Within small venules in the exteriorized cremaster muscle, these studies revealed a glycocalyx thickness on the order of about 0.3–0.4 μm which displaces blood flow from the surface of the endothelium. In contrast, similar applications of PIV to analysis of particle flow over cultured human umbilical vein and bovine aortic endothelial cells revealed hydrodynamically significant thicknesses of only 0.03 and 0.02 μm, respectively (Smith et al. 2003). Thus, in vitro models clearly fail to replicate the in vivo structure of the glycocalyx.

3 Structure of the Glycocalyx

As discussed in Chap. 1, several studies to date have reviewed the structure of the endothelial glycocalyx (Pries et al. 2000; Reitsma et al. 2007; Weinbaum et al. 2007; Chappell et al. 2009a; Gotte 2003). Salient features relevant to WBC adhesion may be summarized as follows. The most prominent components of the glycocalyx are the glycosaminoglycans (GAGs) heparan sulfate (HS), chondroitin sulfate (CS), and hyaluronan (HA). The GAGs HS and CS are covalently

linked to membrane-bound proteoglycans (PGs). Sulfate groups on HS and CS confer a negative charge to these GAGs. The density of GAGs on PGs and glycoproteins varies considerably (Reitsma et al. 2007), and each PG may carry multiple chains of HS and CS, with a ratio of HS/CS of about 4:1 (Rapraeger 1989), and their sulfation level may change depending on the physiological microenvironment (Rapraeger 1989; Vogl-Willis and Edwards 2004). HA does not possess sulfate groups and is not covalently linked to a proteoglycan core protein but is held in place by specific hyaluronan-binding proteins (Laurent and Fraser 1992). In addition to GAG-carrying proteoglycans, adsorbed blood-borne soluble proteins comprise substantial components of the glycocalyx and may be decreased by removing plasma proteins (Adamson and Clough 1992; Huxley and Curry 1991). Under normal physiological conditions, the structure of the glycocalyx layer is stable, and its molecular composition represents a dynamic balance between continued biosynthesis of new glycans and shear-dependent alterations.

Studies of the dimensions and structure of the endothelial glycocalyx have been confounded by the methods of fixation and source of the cells studied (Pries et al. 2000; Reitsma et al. 2007). In vivo observations by direct microscopy have revealed an apparent thickness of the glycocalyx, estimated by the exclusion of erythrocytes and macromolecules (Vink and Duling 1996), on the order of 400–500 nm, which significantly exceeds the dimensions obtained in either fixed specimens or cultured cells. In vitro models with cultured ECs fail to express a glycocalyx of thickness comparable to that found ex vivo (Chappell et al. 2009a). As shown therein, electron microscopy studies of fixed umbilical vein EC revealed a glycocalyx with an average thickness of 878 nm, whereas cultured HUVECs revealed a glycocalyx thickness ranging from only 29 to 118 nm.

Direct measurement of glycocalyx thickness in post-capillary venules by intravital microscopy is technically challenging. In the case of capillaries with single-file motion of RBCs, the width of the red cell column can be easily distinguished from the anatomical capillary diameter (Vink and Duling 1996) to reveal a distance to the EC surface of about 500 μm. As shown therein, infusion of fluorescently labeled 70 kDa dextran (Dx70) revealed a lesser gap between the edge of the fluorescent column and the EC on the order of 400 nm. With cessation of flow, RBCs could be observed to infiltrate the dextran exclusion space completely with zero gap. Mathematical modeling of fluid flow in the glycocalyx suggests that fluid dynamical pressures generated within the glycocalyx can lead to variations of red cell shape and gap width with flow velocity (Feng and Weinbaum 2000; Secomb et al. 2001) that are consistent with in vivo observations (Vink and Duling 1996). Studies of the width of the molecular exclusion zone in capillaries (Vink and Duling 2000) revealed that the edge of the dye column is both charge and molecule size dependent. However, anionic and neutral Dx70 maintained a discrete distance from the EC surface. Application of such techniques has been applied to measure the thickness of the glycocalyx in venules, where WBC-EC adhesion occurs (Gao and Lipowsky 2010). As shown in Fig. 3a for a 35 μm venule, the RBC column is surrounded by a plasma layer that extends to the outer edge of a dark refractive band on the EC surface. Following the infusion of fluorescently labeled Dx70 (Fig. 3b), the edge of the dye column becomes diffuse due to the relatively large diameter and path length along the optical axis. To objectively demarcate the dye exclusion zone, a sigmoidal fit of the radial intensity distribution was made (Fig. 3c) and the edge of the glycocalyx taken as the location of its inflection point. The thickness of the glycocalyx was calculated as the distance between the inflection point and the outer edge of the dark refractive band.

Shown in Fig. 3d are measurements of the thickness of this barrier to infiltration of Dx70 under controlled conditions and following infusion of enzymes to remove specific GAGs from the EC surface. Individually, all three major enzymes significantly reduced the layer thickness below its normal 500 μm level, with heparinase having the greatest effect. A mixture of all three enzymes reduced the layer thickness

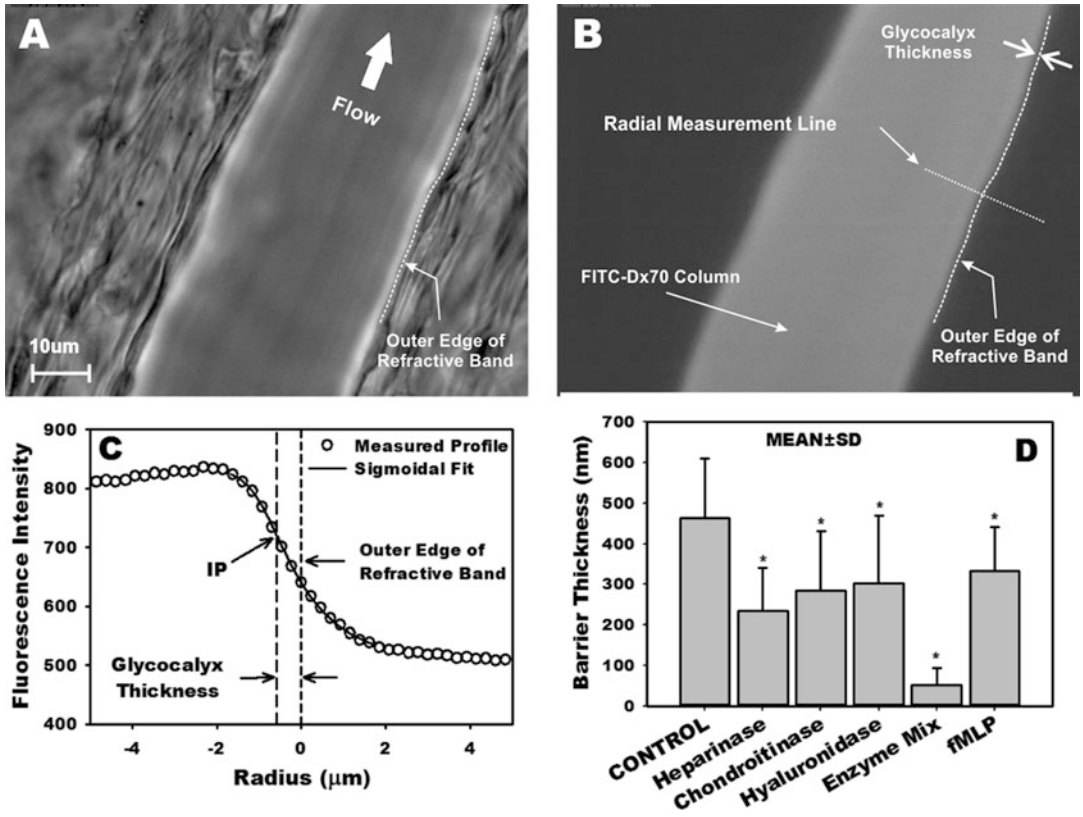

Fig. 3 Measurement of the thickness of the glycocalyx in a 35 μm diameter venule. (**a**) Bright-field view reveals the plasma layer that surrounds the red cell (RBC) core. A dark refractive band can be seen near the EC surface. The outer edge of this band is at the EC surface. (**b**) Circulating fluorescently labeled 70 kDa dextran is shown under fluorescence microscopy and infiltrates the gap between the RBC core and EC surface. (**c**) Radial distribution of dextran fluorescence intensity exhibits a sigmoidal distribution between the RBC column and EC surface (outer edge of the dark refractive band) due to the varying path length along the optical axis. An objective measure of the thickness of the glycocalyx was taken as the distance between the intensity inflection point (IP) and outer edge of the refractive band. (**d**) Thickness was observed to be significantly ($*p < 0.05$) reduced following infusion (by micropipette) with either heparanase, chondroitinase, or hyaluronidase and reduced by 90% with a mixture of all three enzymes. Activation of the endothelium with fMLP significantly reduced thickness due to shedding of glycans. Reproduced from Gao and Lipowsky (2010), with permission

by almost 90%. Accounting for cross-reactivity of the enzymes resulted in (by solving the simultaneous algebraic equations for each GAG) contributions to the thicknesses of the barrier of 43.3, 34.1, and 12.3%, for HS, CS, and HA, respectively. Thus, heparan sulfate appears to represent the major component of the glycocalyx. To simulate changes in the structure of the glycocalyx anticipated in the inflammatory response, the chemoattractant fMLP was topically applied (Fig. 3d) and revealed a significant 28% reduction in glycocalyx thickness that was not significantly different from the losses due to enzymatic cleavage.

Reductions in intensity of lectin staining of the glycocalyx due to fMLP have been correlated with the shedding of glycans (Mulivor and Lipowsky 2004). Loss of glycans has been correlated with increased infiltration of macromolecules in the surface layer in response to the cytokine TNF-α (Henry and Duling 1999, 2000). Quantitative estimates of changes in the

porosity of the ESL have been made by calculation of the diffusion coefficients (D) of the small fluorescent molecule fluorescein isothiocyanate (FITC, 350 Da) by applying a 1-D diffusion model to measurements of radial concentration gradients in the ESL (Gao and Lipowsky 2010). By comparison of measured transients in radial intensity of a bolus of FITC with that of a computational model, a diffusion coefficient D was obtained. Values of D were obtained corresponding to the thickness of the layer demarcated by Dx70 (D_{Dx70}), and a smaller sublayer 173 nm above the EC surface (D_{173}), prior to and following enzyme infusion and superfusion with fMLP. The magnitude of D_{Dx70} was twice that of D_{173} suggesting that the glycocalyx is more compact near the EC surface. Chondroitinase and hyaluronidase significantly increased both D_{Dx70} and D_{173}. However, heparinase decreased D_{Dx70} and did not induce any significant change for the D_{173}. These observations suggest that the three GAGs are not evenly distributed throughout the glycocalyx and that they each contribute to permeability of the glycocalyx to a differing extent.

4 Shedding of the Glycocalyx

Functional changes in the barrier formed by the ESL have been observed in response to a broad spectrum of agents. Topical stimulation of the endothelium for prolonged periods (20–120 min) with the cytokine TNF-α results in an increased porosity of the glycocalyx in the absence of WBC-EC adhesion (Henry and Duling 2000). Significant shedding of components of the glycocalyx in coronary vessels has been observed following perfusion of isolated hearts for 20 min with TNF-α, which was lessened by the serine protease inhibitor antithrombin III (Chappell et al. 2009b). Acute activation of the endothelium in post-capillary venules with the chemoattractant fMLP induced a rapid (<5 min) shedding of glycans from the EC surface as evidenced by a loss of lectin-laden microspheres bound to the EC surface (Mulivor and Lipowsky 2004). Shedding of proteoglycans and GAGs from cultured endothelial cells, or their analogs, occurs in response to a broad spectrum of agonists (Park et al. 2000; Colburn et al. 1994; Fux et al. 2009; Ihrcke et al. 1993; Platt et al. 1990, 1991; Fitzgerald et al. 2000). Shedding of heparan sulfate proteoglycans (namely, the ectodomain of syndecans 1–4) occurs in response to endotoxin (Colburn et al. 1994), serine and/or cysteine proteinases (Ihrcke and Platt 1996), complement activation (Platt et al. 1991), thrombin and growth factors (Subramanian et al. 1997), and activation of protein tyrosine kinase by phorbol ester (Fitzgerald et al. 2000). Using hydroxamic acid inhibitors of matrix metalloproteinases, it has been shown that proteolytic cleavage of the syndecan ectodomain results from the convergence of multiple intracellular pathways that activate a cell surface metalloproteinase (Fitzgerald et al. 2000).

In vivo, shedding of the endothelial glycocalyx has been found in response to inflammation (Mulivor and Lipowsky 2004; Henry and Duling 2000), hyperglycemia (Zuurbier et al. 2005), endotoxemia and septic shock (Hofmann-Kiefer et al. 2009), presence of oxidized LDL (Constantinescu et al. 2001), TNFα (Chappell et al. 2009b), atrial natriuretic peptide (Bruegger et al. 2005), abnormal blood shear stress (Gouverneur et al. 2006; Haldenby et al. 1994), ischemia-reperfusion injury (Mulivor and Lipowsky 2004), light-induced production of free radicals (Vink and Duling 1996), and bypass surgery (Rehm et al. 2007; Svennevig et al. 2008). These observations have led to an underlying connection between integrity of the glycocalyx and vascular homeostasis (Mulivor and Lipowsky 2004; Zuurbier et al. 2005).

Shedding of the glycocalyx in response to cytokines and chemoattractants occurs in all three principal divisions of the microvasculature: arterioles (Henry and Duling 2000), capillaries (Constantinescu et al. 2001; Henry and Duling 2000), and venules (Mulivor and Lipowsky 2004; Henry and Duling 2000). To illustrate, shown in Fig. 4 is the intensity of glycans in the ESL stained with a fluorescently labeled lectin (BS-1) in the three principal divisions of the mesenteric microvasculature (rat) (Lipowsky et al. 2011).

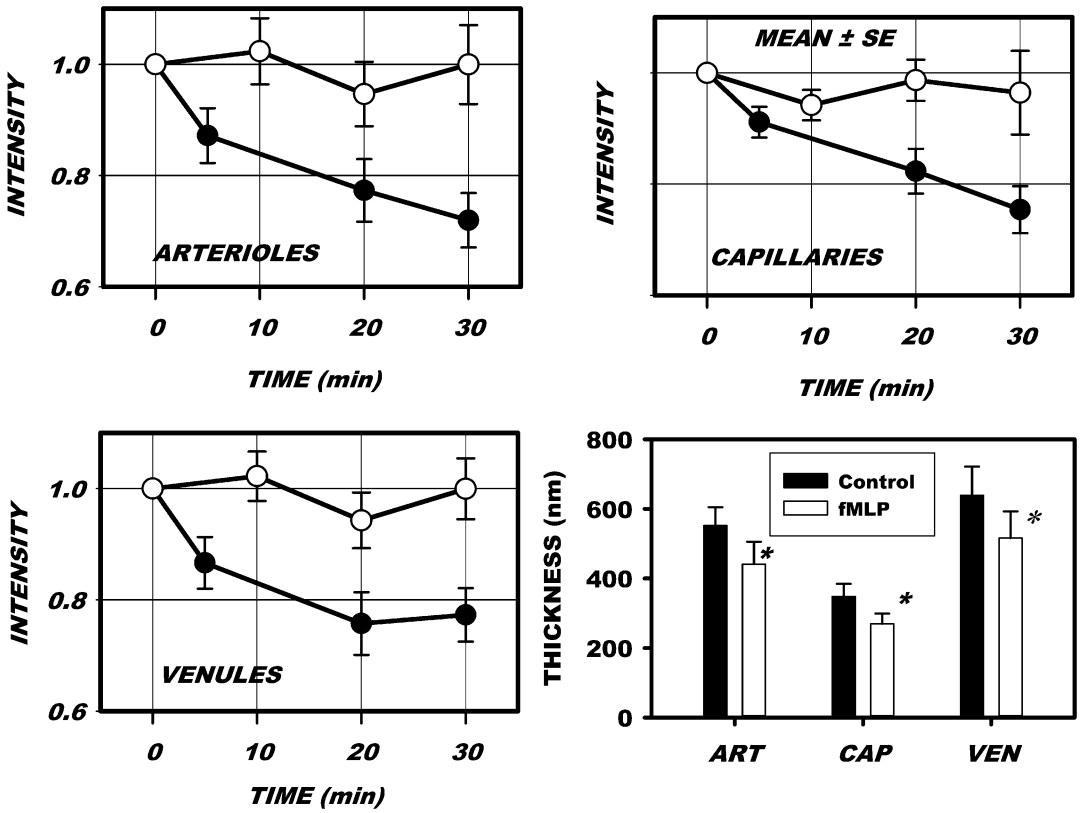

Fig. 4 Shedding of glycans on the EC surface of post-capillary venules in mesentery (rat) in response to topical application of the chemoattractant fMLP (10^{-7} M) compared to control values obtained with superfusion of the tissue with Ringer's solution. Glycan concentration was assumed proportional to the intensity of fluorescently labeled lectin (BS-1) on the EC surface and normalized to initial values. Shown are mean values ± SE for arterioles, capillaries, and venules. Intensity of the lectin stain falls rapidly within the first 5 min of onset of the fMLP and steadily decreases during the entire observation period. After 30 min exposure to fMLP, thickness of the glycocalyx decreased significantly ($*p < 0.05$). Data are mean ± SE. From Lipowsky et al. (2011), with permission

The normalized intensity is shown under resting conditions and following topical application of the chemoattractant fMLP (10^{-7} M). About 30% of all lectin-stained glycans were shed from the EC during a 30 min exposure to fMLP. This shedding appears to be accompanied by a significant reduction in thickness in each division (Fig. 4d). Although it has been postulated that such reductions in thickness are insufficient to expose WBC adhesion receptors (Marki et al. 2015), concomitant increases in porosity (Henry and Duling 1999, 2000) and deformability (Padberg et al. 2014; Wiesinger et al. 2013) of the ESL may promote WBC infiltration and adhesion during inflammation.

5 Enzymatic Cleavage of the Glycocalyx

With the majority of WBC adhesion receptors situated in post-capillary venules, as, for example, in the case of ICAM-1 (Iigo et al. 1997), shedding of the venular glycocalyx may play an important role in the inflammatory process. The cellular signaling cascades resulting from pathological conditions and initiating shedding of the glycocalyx are not fully understood. However, direct in situ observations of shedding in post-capillary venules suggest that several key enzymes may be responsible for shedding of the glycocalyx components (Mulivor and Lipowsky

2009). Matrix metalloproteases (MMPs) on the surface of the venular endothelium are rapidly activated by superfusion of the mesenteric tissue with fMLP and may be inhibited by superfusion with subantimicrobial doses (0.5 μM) of the antibiotic doxycycline (Mulivor and Lipowsky 2009). The inhibitory activity of doxycycline on shedding results from its direct effect on MMP activation and not by its ability to chelate divalent cations (Lipowsky et al. 2011), as evidenced by inhibition of MMP activation by the zinc-chelating hydroxamic acid inhibitor GM6001, and lack of inhibition by chelation of cations with EDTA. The possible role of doxycycline as a scavenger of reactive oxygen species (ROS) has been raised (Golub et al. 1998). However, direct evidence that ROS cause shedding in response to chemoattractants or cytokines remains to be obtained. In addition, MMP inhibition has been shown to have no effect on ROS-induced shedding (Lipowsky and Lescanic 2013).

Fluid shear stresses acting on the EC surface may affect the structure of the glycocalyx by either disrupting molecular constituents, affecting biosynthesis of new components, or activating proteases and lyases synthesized by the endothelium (Mulivor and Lipowsky 2004; Arisaka et al. 1995). Increased synthesis of GAGs by cultured monolayers of ECs occurs with prolonged exposure to high shear stresses of 15 or 40 dyn/cm^2 (Arisaka et al. 1995). These results were in contrast to prior studies that revealed a decrease in proteoglycan synthesis when ECs were cultured under low levels of shear stress (Grimm et al. 1988). In vivo studies of the accumulation of glycans on the surface of post-capillary venules during a 1 h period of ischemia demonstrated a 15–40% increase in glycan content on the surface of the EC (Mulivor and Lipowsky 2004). Upon reperfusion of these venules, this excess of surface glycans was washed out, and glycan levels (indicated by accumulation of lectins on the EC surface) momentarily fell below pre-ischemic (control) conditions before returning to normal levels. This postischemic fall below pre-ischemic levels was inhibited by superfusion of the tissue with pertussis toxin, thus suggesting a G-protein-mediated activation of enzymatic cleavage of GAGs and/or proteoglycans on the EC surface.

The hypothesis that matrix metalloproteinases (MMPs) may alter the endothelial glycocalyx and thus facilitate shedding under pathological conditions is well supported. Matrix metalloproteinases represent a family of over two dozen zinc-dependent proteases that play a role in normal tissue remodeling during bone growth, wound healing, reproduction, cancer, inflammation, and cardiovascular disease (Spinale 2007). MMPs (−1 and − 9) serve to cleave the endothelial insulin receptor and CD18 on leukocytes in the spontaneously hypertensive rat (DeLano and Schmid-Schonbein 2008). Oxidative stress in the diabetic heart may activate MMP-2 and lead to the development of diabetic cardiomyopathy (Yaras et al. 2008). Modification of the extracellular matrix by MMPs has been shown to be a critical step in angiogenesis (Haas et al. 2000) and atherosclerosis (Li et al. 1996). MMP-2, MMP-7, and MMP-9 were shown to be capable of directly cleaving chondroitin sulfate (Gronski et al. 1997). In addition, MMP-1 was shown to cleave the heparan sulfate proteoglycan syndecan-1 (Endo et al. 2003). MMPs can be stored within and released by the endothelium. It has been shown (Taraboletti et al. 2002) that both the active and proactive forms of MMP-2 and MMP-9 are stored in vesicles within the EC and both forms of MMP-7 have a high affinity for and bind to heparan sulfate (Yu and Woessner Jr 2000). Therefore, mechanisms exist by which MMP's may be rapidly released by endothelial cells. Innate inhibition of MMPs is derived from tissue inhibitors of metalloproteinases (TIMPs), a family of four different molecules made unique by their expression, localization, and inhibitory activity. Much like the MMPs, TIMPs are capable of binding heparan sulfate and chondroitin sulfate in the glycocalyx (Yu and Woessner Jr 2000).

The putative role of MMPs in cleaving glycans from the EC surface is supported by studies of in situ microzymography to quantify MMP activation on the surface of post-capillary venules (Mulivor and Lipowsky 2009). Hence,

it is likely that cleavage of GAG-bearing proteoglycans by either membrane-bound or cytosolic MMPs in the endothelial cell may be responsible for shedding of the glycocalyx. This hypothesis is also supported by studies of syndecan-1 shedding from human embryonic kidney cells caused by membrane type matrix metalloproteinase-1 (MT1-MMP) (Endo et al. 2003), shedding of syndecan-1 and syndecan-4 from HeLa tumor cells by MMP-9 (Brule et al. 2006), shedding of syndecan-1 from pancreatic carcinoma cells by MMP-7 (Ding et al. 2005), and shedding of syndecan-1 by MMP-7 during transmigration of neutrophils from the interstitium to alveoli in the lung (Li et al. 2002).

The effects of MMP inhibition on glycan shedding and WBC-EC adhesion in response to topical application of fMLP is illustrated in Fig. 5. Without MMP inhibition, fMLP induces a rapid (<10 min) shedding of glycans and an eightfold increase in the number of WBCs adhered to the walls of post-capillary venules (Mulivor and Lipowsky 2009). Superfusing the tissue with 0.5 μM. doxycycline results in a significant attenuation of glycan shedding and WBC-EC adhesion.

Whereas MMP activity presumably cleaves the protein core of GAG-bearing proteoglycans, cleavage of GAG chains by EC secretion of heparanase may also contribute to shedding (Chappell et al. 2008; Becker et al. 2015). Heparin has long been recognized as an inhibitor of heparanase activity (Bar-Ner et al. 1987), and considerable experimental and clinical evidence supports its anti-inflammatory activity (Page 2013). The anti-inflammatory properties of unfractionated and low-molecular-weight heparins (LMWH) have been studied extensively, although precise mechanisms have not been established (Oduah et al. 2016). Several studies have aimed to delineate the role of heparin in WBC-EC rolling and adhesion, and transmigration trough the microvessel wall. Treatment with LMWH dramatically diminished sepsis-induced neutrophil sequestration in the lung (Ning et al. 2015) and attenuated shedding of the glycocalyx in septic shock (Yini et al. 2015). Heparin has been reported to diminish or protect against reperfusion injury in various animal models (Young 2008). Intradermal administration of heparin attenuated eosinophil accumulation in response to inflammatory stimuli in a dose-dependent manner (Teixeira and Hellewell 1993). Binding of heparins to selectins (Koenig et al. 1998), WBCs (Page 2013; Diamond et al. 1995; Lever et al. 2000), and HSPGs and other constituents of the EC glycocalyx (Nordling et al. 2015; VanTeeffelen et al. 2007) has been shown to inhibit the inflammatory process. Use of low-molecular-weight heparin fractions has shown potential for protecting the endothelial glycocalyx from degradation in nephrosis (Gaddi et al. 2010), diabetes (Eskens et al. 2013), thrombosis (Daniels et al. 2006), retinal neovascularization (Jo et al. 2014), and inflammation (Becker et al. 2015; Kolarova et al. 2014). These studies found that the agent sulodexide, a mixture of low-molecular-weight heparin and dermatan sulfates, afforded significant protection of the glycocalyx from degradation.

The effects of graded concentrations of LMWH (Lovenox®, relative molecular mass, Mr. = 4500) on shedding of glycans and WBC-EC adhesion (Lipowsky and Lescanic 2017) are illustrated in Fig. 5b, d. With increasing doses of LMWH up to 0.22 mg/kg, competitive binding of LMWH to heparanase may attenuate the initial shedding of glycans due to fMLP. With greater doses (>0.6 mg/kg), glycan concentration appears to rise due to a compaction of the glycocalyx as HS chains are cleaved and the layer collapses. Eventually, the inhibition of shedding by LMWH is overwhelmed by activity of either MMPs, heparanase, or other sheddases. The WBC-EC adhesion response to fMLP (Fig. 5d) was significantly attenuated during the initial 10 min at the highest dose of LMWH but eventually rose with prolonged exposure to the chemoattractant. In these studies, WBC adhesion correlated with intensity of the lectin stain for all measurements and revealed a significant 40% reduction in adhesion as intensity increased 50%. This relationship was attributed to LMWH inhibition of heparanase and/or binding to

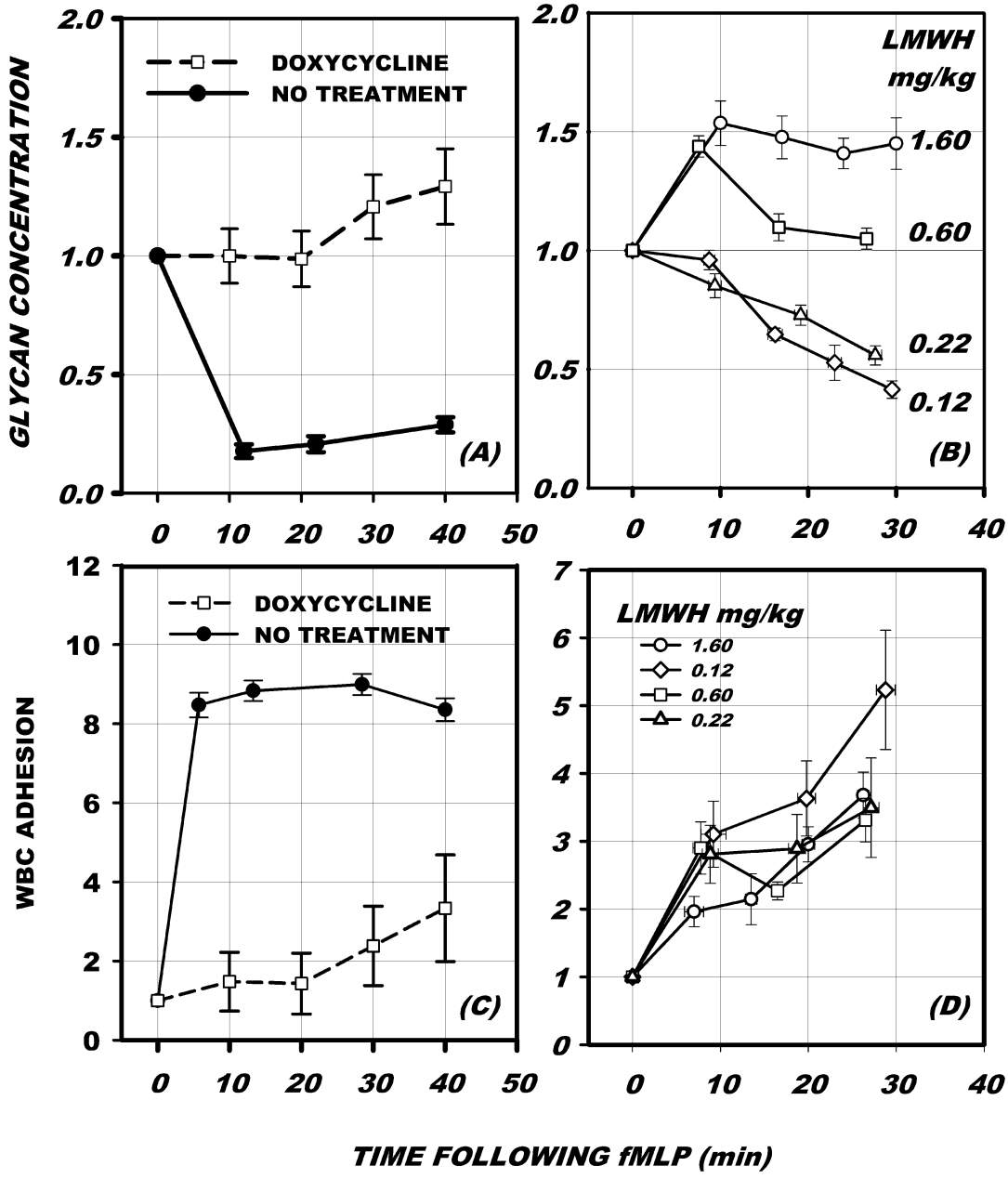

Fig. 5 Enzymatic shedding of the glycocalyx and WBC-EC adhesion in mesenteric venules. Glycan concentration was taken in proportion to the intensity of fluorescently labeled lectin (BS-1), normalized to initial values as a function of time following topical application of fMLP. (**a**) Glycan concentration in the glycocalyx fell dramatically following onset of fMLP stimulation. This fall was completely abolished by superfusion of the tissue with the MMP inhibitor doxycycline at a concentration of 0.5 μM. (**b**) Glycan concentration due to fMLP following IV administration of low-molecular-weight heparin (LMWH) of the indicated doses. Low doses (0.22 mg/kg) inhibited the initial fall during the first 10 min. High doses (>0.60 mg/kg) resulted in a rise in glycan concentration presumably due to ligation of HS chains and subsequent collapse of the glycocalyx. (**c**). The WBC-EC adhesion response to fMLP, with and without superfusion with doxycycline. (**d**) WBC-EC adhesion with infusion of LMWH. The high dose of LMWH initially delayed the rise in WBC-EC adhesion. Data are means ± SE. (**a**) and (**c**) redrawn from Mulivor et al. (Atherton and Born 1972); (**b**) and (**d**) redrawn from Lipowsky et al. (Atherton and Born 1973)

components of the glycocalyx with a resultant mitigation of glycan shedding, compaction of the lectin stain, and stabilization of the glycocalyx.

6 Leukocyte Rolling and Adhesion

Based upon the rolling and adhesion of WBCs on either artificial surfaces coated with receptors for specific ligands (Alon et al. 1995; Lawrence and Springer 1991, 1993) or monolayers of cultured endothelial cells (Arisaka et al. 1995; Hoover et al. 1980; Lawrence et al. 1987), it has long been held that adhesiveness is governed by regulation of the affinity and avidity of the integrin molecules on the WBC and EC (Zarbock and Ley 2009; Kinashi and Katagiri 2004; Laudanna et al. 2002; Luo et al. 2007). In vivo studies of post-capillary venules (Arfors et al. 1987; House and Lipowsky 1987b; Ley et al. 1995) have supported this concept. In addition, the mechanical properties of the glycocalyx may play a role in the adhesion process in light of the ability of microvilli on the surface of rolling WBCs to penetrate the surface layer to reach adhesion receptors (Zhao et al. 2001). WBC microvilli may range in length from 0.3 to 0.7 μm (Weinbaum et al. 2007). The ability to penetrate the glycocalyx may depend on changes in porosity and stiffness attendant to physiological stimuli (Weinbaum et al. 2007; Platts et al. 2003; Platts and Duling 2004). Under normal conditions, the apparent thickness of the glycocalyx significantly exceeds the lengths of endothelial cell (EC) receptors involved in leukocyte (WBC) rolling on the EC (selectins) and firm adhesion to the EC (integrins). The lengths of these receptors range from 20 nm for the β2 integrin ligands to 30–40 nm for E- and P-selectins (Springer 1990). Thus, reduction in thickness of the glycocalyx by either chemoattractants (e.g., fMLP) (Gao and Lipowsky 2010) or cytokines (e.g., TNF-α) (Henry and Duling 2000) accompanied by increased porosity may enhance access to adhesion receptors on the EC surface. It has been shown that perfusion of post-capillary venules with heparinase or superfusion of the tissue with fMLP served to increase binding of circulating antibodies to ICAM-1 on the EC surface (Mulivor and Lipowsky 2002). Although in this study, firm adhesion of WBCs was not stimulated by perfusion of venules with heparinase, most likely because substrates for leukocyte rolling were also removed, subsequent studies using heparitinase (which may cleave less sulfated heparan sulfate chains) produced an increase in firm WBC adhesion (Constantinescu et al. 2003). Alternatively, it has been demonstrated that heparinase reduces stimulated rolling and adhesion of WBCs in post-capillary venules by inhibiting externalization of P-selectin and/or compromising the structural interactions between heparan sulfate proteoglycans and selectins (Hayward et al. 1998).

Changes in the glycocalyx attendant to EC activation also affect WBC rolling adhesive interactions with the EC. Taking the rolling velocity of WBCs (normalized with respect to estimated wall shear rates, S.R.) as a measure of the adhesiveness of the EC surface (the lower the ratio of V_{WBC}/SR, the greater the adhesiveness), it was found that superfusion of the tissue with inhibitors of MMP activity, such as doxycycline or the zinc chelator GM6001, decreased the rolling velocity, thus promoting adhesiveness of the EC surface during WBC rolling. These results suggest that there is a basal level of sheddase activity on the EC surface that may be suppressed by MMP inhibition, which leads to an excessive accumulation of adhesion receptors on the EC surface that retard the rolling motion of WBCs. The presence of a basal level of MMP activity on the EC surface of post-capillary venules has been demonstrated by measuring the fluorescence activity of fluorescence substrates circulating in the plasma, which is reduced with MMP inhibition (Mulivor and Lipowsky 2009). Interestingly, superfusion of the tissue with fMLP alone causes a similar reduction in rolling velocity, presumably due to a combination of conformational changes in adhesion receptors on the EC surface and enhanced externalization of adhesion receptors (e.g., P-selectin) and shedding of the glycocalyx. While inhibition of MMP activation and activation with fMLP both resulted in diminished rolling velocity, the strength of the

adhesive bond during firm adhesion was found to be less with MMP suppression compared to that with fMLP, presumably due to conformational changes of WBC integrin receptors induced by fMLP (Lipowsky et al. 2015).

Similar adhesive interactions have also been observed, in part, by experiments in other tissues and cells. Inhibition of L-selectin shedding from WBCs by the metalloprotease inhibitor KD-IX-73-4 was found to reduce WBC rolling velocity in post-capillary venules of hamster cremaster muscle (Hafezi-Moghadam et al. 2001), which was attributed to inhibition of L-selectin shedding on the leukocyte alone. Although KD-IX-73-4 had no apparent effect on the endothelial glycocalyx, these studies bring to light the potential for metalloprotease inhibition to affect leukocyte rolling and adhesion. Further, comparison of the inhibitory activity of KD-IX-73-4 on the shedding of the endothelial protein C receptor (EPCR) from EA.hy926 endothelial cells, with inhibition of the MMP inhibitor GM6001, revealed that the latter was ineffective in inhibiting the release of EPCR (Xu et al. 2000). Thus, if this endothelial selectivity of the MMP inhibitors GM6001 and doxycycline applies to the reductions of WBC rolling velocity in venules, then the role of MMP inhibition on affecting the adhesive properties of the endothelial glycocalyx is further supported. It has also been shown that reduced rolling velocity of WBCs occurs following exposure of cremaster venules to TNF-α (Jung et al. 1998). Although these results were attributed to conformational changes of adhesion receptors in response to TNF-α, this trend may reflect the shedding of glycans from the EC surface, as noted previously (Henry and Duling 2000), and the enhanced access to adhesion ligands (e.g., ICAM-1).

7 Conclusions

In summary, the endothelial surface layer, which consists of the EC glycocalyx and a layer of adsorbed proteins, has been implicated as a barrier to WBC-EC adhesion. The primary glycoproteins are decorated with the principal glycosaminoglycans (GAGS) heparan sulfate (HS) and chondroitin sulfate (CS) that are encased in a meshwork of hyaluronic acid (HA). Together, these glycans form a layer that can be observed in vivo on the surface of the EC that is on the order of 500 μm thick. Measurement of the precise thickness of the ESL is fraught with many difficulties and is most easily implemented by exclusion of macromolecules from the EC surface. In vitro, the ESL is much smaller, on the order of 1/10th the in vivo thickness. Staining of glycans with carbohydrate-binding proteins (lectins) permits visualization of the ESL and reveals that the composition of the glycocalyx readily changes during the inflammatory process, as evidenced by the shedding of glycans with activation of the endothelium by chemoattractants and cytokines. Reductions in thickness and increases in porosity of the ESL due to an inflammatory stimulus facilitate greater access to WBC adhesion receptors on the EC surface and thus promote WBC-EC adhesion.

The effectors of ESL shedding are most likely members of the family of metalloproteinases, which cleave the core proteoglycans, and endoglycosidases (namely, heparanase), which cleave GAGs attached to the core proteins. Direct intravital microscopic studies of shedding of the glycocalyx have suggested that glycan shedding may be inhibited by MMP inhibitors and competitive binding to EC-derived heparanase. The relative proportions of MMP and heparanase-induced shedding remain to be fully delineated, as well as the extent to which MMPs and heparanase affect their mutual release and activation. Identification of the specific proteases responsible for shedding is further complicated by the ability of specific MMPs to activate other members of the MMP family and for other proteases to activate MMPs. There is also an indirect association between heparanase and MMP expression (Purushothaman et al. 2008, 2011, 2010; Zcharia et al. 2009). For example, blocking activation of MMP-9 inhibited heparanase-induced syndecan-1 shedding in myeloma cells (Purushothaman et al. 2010), and overexpression of heparanase in cultured human mammary carcinoma cells resulted in diminished expression of MMP-2,

MMP-9, and MMP-14 (Zcharia et al. 2009). It is clear, however, that stabilization of the glycocalyx during inflammation may indeed mitigate WBC-EC adhesion. Thus, the development of new strategies to directly target shedding of the glycocalyx may have significant therapeutic value.

Acknowledgments This work was supported in part by NIH R01 HL-39286.

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