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Protease Activity and the Role of the Endothelial Glycocalyx in Inflammation

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Abstract

A new paradigm for governance of leukocyte-endothelium (WBC-EC) adhesion during the inflammatory process is presented in which shedding of the endothelial glycocalyx exposes adhesion molecules on the EC surface, which promotes WBC-EC adhesion. It is postulated that the effector of this shedding is the activation of extracellular proteases, one of which may be a member of the matrix metalloproteinase (MMP) family of zinc dependent endopetidases. This model for the role of the glycocalyx as a barrier to WBC-EC adhesion includes the additional participation of normally active extracellular proteolytic enzymes, i.e. sheddases, which may cleave proteoglycans or activate lyases that cleave GAG chains in the glycocalyx. In support of this hypothesis, studies are examined which have established the concurrent activation of MMP proenzymes on the EC surface, shedding of the glycocalyx, and enhanced WBC-EC adhesion.

Introduction

Leukocyte interactions with the endothelium during the inflammatory process have long been recognized to occur in a sequence of events that depend on hemodynamic forces that transport leukocytes (white blood cells, WBCs) to and from the endothelial cell (EC) surface, and receptor-ligand interactions that govern the strength of the adhesive bonds between WBC and EC. Following radial migration (margination) of WBCs to the EC surface as blood flow exits the capillary network into post capillary venules, WBCs saltate and roll along the EC. Prolonged WBC rolling contact is facilitated by the “selectin” family of adhesion receptors, and subsequent firm adhesion to the EC is mediated by the “integrin” family of receptors as a prelude to their migration through the vascular wall. Extensive reviews of these processes have highlighted the adhesion molecules involved [1–4]. In most of these studies, it was assumed that the rapid adhesion of WBCs to the EC in response to a stimulus resulted from conformational changes of receptors on the EC [1] and that adhesiveness was influenced by regulation of the affinity and avidity of the integrin molecules on the WBC and EC [1–4]. An upregulation of “affinity” was associated with conformational changes in receptors and/or ligands, which increases ligand binding and decreases ligand dissociation [1]. These observations were derived from *in vitro* studies of the rolling and adhesion of WBCs on either artificial surfaces coated with receptors for specific ligands [5–7] or monolayers of cultured endothelial cells [8–10], and *in vivo* studies of post-capillary venules in the living animal [11–13]. While such studies have contributed

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greatly to understanding the strength of WBC-EC adhesive interactions and the dynamics of WBC sequestration and extravasation, they have ignored the effects of steric hindrance on receptor-ligand bonding that may arise from proteoglycans and polysaccharides that surround the adhesion receptors.

The Glycocalyx

The luminal surface of endothelial cells is coated with a carbohydrate-rich surface layer consisting of adsorbed proteins and membrane bound proteoglycans, collectively referred to as the endothelial glycocalyx [14; 15]. The glycocalyx is an important barrier to transvascular exchange of water and solutes [16; 17] and sieving of plasma-borne macromolecules [18–20]. It has been shown to be a physiologically significant binding site for antithrombin III, tissue factor pathway inhibitors, lipoprotein lipase, vascular endothelial growth factor, fibroblast growth factor, and extracellular superoxide dismutase [15]. Recent studies have shown that the glycocalyx serves as a barrier to leukocyte-endothelium adhesion [21], shear stress sensor and regulator of mechanotransduction [22].

The fine structure of the glycocalyx and its potential impact on permeability has been explored using techniques of electron microscopy [23; 24], intravital microscopy [17; 20] and mathematical modeling [25–27]. It has been suggested that the glycocalyx is a porous layer composed of a matrix of molecules that are arranged in a regular pattern [24]. The most prominent components of the glycocalyx are the glycosaminoglycans (GAGs) heparan sulfate (HS), chondroitin sulfate (CS) and hyaluronan (HA). Both HS and CS are synthesized inside the Golgi apparatus, whereas HA synthases (HASs) are plasma membrane associated such that newly synthesized HA strands are extruded outside the cell [28]. There are several ways for HA to be integrated into the glycocalyx. The primary HA receptor in the glycocalyx is thought to be CD44 [29] along with HA binding protein [30]. HA chains can be self-assembled by steric interaction into random fibrous networks [31]. The principal proteins on the EC surface that bind HS and CS to form the proteoglycans are the transmembrane syndecans and the membrane-bound glypicans [14]. The most prevalent proteoglycan, syndecan-1, is one of four members of this family of proteoglycans and is a type I integral membrane protein composed of an intracellular domain, a transmembrane domain, and an extracellular core [32]. Syndecan-1 typically has one to three HS and one to three CS GAG molecules attached to its core protein. Besides GAG-carrying proteoglycans, blood-borne soluble proteins are also important components of the glycocalyx, which have been shown experimentally to be decreased by removing plasma proteins [33; 34]. Under normal physiological conditions, the structure of the glycocalyx layer is fairly stable with a molecular composition that represents a dynamic balance between biosynthesis of new glycans and shear dependent removal of existing constituents [35], as schematized in Fig. 1. Fluid shear stresses acting on the EC surface may affect the structure of the glycocalyx by either disrupting its molecular constituents or activation of proteases and lyases synthesized by the endothelium [8; 35].

The apparent thickness of the glycocalyx has been estimated by the exclusion of erythrocytes and macromolecules [36] to be on the order of 400–500 nm, which 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). Studies of the lengths of these receptors have shown a range from 20 nm for the $\beta 2$ integrin ligands to 30–40 nm for E- and P-selectins [37]. Thus, it has been postulated that WBC-EC adhesion is strongly dependent upon the thickness and composition of the glycocalyx [35]. In this context, it has been shown that enzymatic removal of HS with heparinase infusion in post-capillary venules, or stimulation with chemoattractants results in an increased exposure of $\beta 2$ integrin ligand ICAM-1 and elevated WBC adhesion as illustrated in Fig. 2 for the case of stimulation of shedding with the chemoattractant f-met-leu-phe. Concurrent shedding of

the glycocalyx, as evidenced by its diminished thickness, and increased WBC-EC adhesion have been shown in response to activation of the EC with fMLP. Both glycan shedding and WBC adhesion have been attenuated by inhibition of MMP activity with sub-antimicrobial doses of doxycycline [38].

Shedding of the Glycocalyx

Shedding of proteoglycans and GAGs from cultured endothelial cells, or their analogs, has been studied in response to a broad spectrum of agonists [39–45]. Shedding of heparan sulfate proteoglycans (namely the ectodomain of syndecans 1–4) has been shown to occur in response to endotoxin [40], serine and/or cysteine proteinases [46], complement activation [44], thrombin and growth factors [47] and activation of protein tyrosine kinase by phorbol esters [45]. 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 [45].

In vivo, the endothelial glycocalyx has been shown to be shed in response to inflammation [35; 48], hyperglycemia [49], endotoxemia and septic shock [50], presence of oxidized LDL [51], TNF α [52], atrial natriuretic peptide [53], abnormal blood shear stress [54; 55], ischemia-reperfusion injury [35], and during by-pass surgery [56; 57]. These observations have lead to the hypothesis of an underlying connection between integrity of the glycocalyx and vascular homeostasis [35; 49]. Shedding of the glycocalyx in response to cytokines and chemoattractants has been shown to occur in all three principal divisions of the microvasculature: arterioles [48], capillaries [48; 51] and venules [35; 48]. With the majority of WBC adhesion receptors being situated in the post-capillary venules, as for example in the case of ICAM-1 [58], shedding of the venular glycocalyx may play an important role in the inflammatory process. The exact cellular signaling cascades between pathological conditions and shedding of the glycocalyx are not yet fully understood. However, direct *in situ* observations of shedding in post-capillary venules suggests that several key enzymes, such as the matrix metalloproteinases, are directly responsible for shedding of the glycocalyx components [38].

Protease Activation and Leukocyte-Endothelium Adhesion

Considerable evidence suggests that matrix metalloproteinases (MMPs) have the ability to modify components of the endothelial glycocalyx and thus facilitate the shedding of its constituents under pathological conditions. 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 [59]. Recent studies suggest that MMPs (-1 and -9) serve to cleave the endothelial insulin receptor and CD18 on leukocytes in the spontaneously hypertensive rat [60]. Modification of the extracellular matrix by MMPs has been shown to be a critical step in angiogenesis [61] and atherosclerosis [62]. MMP-2, MMP-7 and MMP-9 were shown to be capable of directly cleaving chondroitin sulfate [63]. In addition, MMP-1 was shown to cleave the heparan sulfate proteoglycan syndecan-1 [64]. MMPs can be stored within and released by the endothelium. It has been shown [65] that both the active and proactive forms of MMP-2 and MMP-9 are stored in vesicles within the EC. Similarly, MMP-7, in both its constitutive and latent forms, has a high affinity for and binds to heparan sulfate [66]. This suggests that mechanisms exist by which MMP's can be rapidly released by endothelial cells without the need to wait for upregulation of mRNA. Inhibition of MMPs occurs naturally by a class of tissue inhibitors of metalloproteinases (TIMPs). TIMPs are comprised of 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 [66]. While the specific target metalloproteinase responsible for

proteolytic cleavage remains to be identified, it has been suggested that TIMP-3, but not TIMP-1 or -2, inhibit shedding of the syndecan ectodomain [45]. Co-regulation of heparanase and MMPs have been found in heparanase knock-out mice, where an increase in MMP activity appeared to compensate for a loss of heparanase expression [67], thus suggesting a yet to be explored linkage between disruption of GAGs and cleavage of the core proteins by protease activity.

As summarized in Fig. 3, inhibition of MMP activity by either doxycycline or the hydroxamate zinc chelator GM6001 significantly reduces the velocity at which WBCs roll along the EC [68]. Analysis of the balance of forces that tend to sweep WBCs from the EC and remove them by disruption of WBC-EC adhesive bonds suggests that reduction in the ratio of V_{WBC} to hydrodynamic shear rates at the wall ($\dot{\gamma}$) is inversely proportional to the strength of the WBC-EC adhesive bond. Thus, inhibition of basal MMP activity at the EC surface may result in an increased level of adhesion molecules (selectins) which causes a reduction in rolling velocity. Interestingly, increased adhesiveness also follows shedding of the glycocalyx and exposure of integrin receptors (ICAM-1) in response to the chemoattractant fMLP [21].

In Vitro vs. In Vivo Models

The preceding findings suggest that experimental models of the inflammatory process must replicate not only the adhesive interactions between WBCs and ECs but also the lability, composition and structure of the endothelial surface layer. *In vitro* models with cultured ECs fail to express a glycocalyx of thickness comparable to that found *ex vivo* [69]. As shown therein, electronmicroscopy 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 [69]. Earlier studies also suggested a disparity in vascular permeability between cultured ECs and the *in situ* microvascular wall, as evidenced by a two-fold greater flux of albumin across cultured cells compared to that in capillaries [70], which may be attributed in part to differences in the structure of the overlying glycocalyx.

The presence of the glycocalyx has been implicated as a factor affecting the mechanics of blood flow in capillaries where its significant thickness may lessen the effective lumen available for red cell transport [71]. Enzymatic removal of the glycocalyx within the microvasculature by infusion of heparinase has been shown to correlate with a decrease in the resistance to flow [72]. Existence of 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) [73]. Within small venules in the exteriorized cremaster muscle, these studies have revealed a glycocalyx thickness on the order of 0.5 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. Thus, *in vitro* models clearly fail to replicate the *in vivo* structure of the glycocalyx.

Summary and Conclusions

These observations of WBC-EC interactions lead to the hypothesis that by addressing the structure and function of the endothelial glycocalyx, intervention in the inflammatory process may be made by taking advantage of the protective barrier offered by the endothelial glycocalyx. Studies have shown that the thickness and composition of the glycocalyx results from an equilibrium between continued biosynthesis of new constituents (membrane bound proteoglycans and GAGs), which may be shed in response to the activation of proteases, such as the family of matrix metalloproteinases, and lyases such as heparanase produced by

endothelial cells. The adhesive interactions between WBCs and ECs are a function of the composition of the glycocalyx, whereby the rolling and firm adhesion may be affected by protease activity. Cleavage of components of the glycocalyx, by MMP activity, for example, may unmask adhesion receptors on the EC surface and promote WBC rolling and firm adhesion. In contrast, inhibition of basal MMP activity may have a similar effect by allowing for an accumulation of ligands that promote WBC rolling and a greater probability of WBC-EC adhesion. Thus, stabilization of the glycocalyx through the normalization of protease activity may be of benefit in mitigating the inflammatory process [74].

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Figure 1.

Schematized view of the endothelial glycocalyx. The composition of the glycocalyx reflects a balance of continued biosynthesis of glycans and its shear dependent removal. It is hypothesized that proteases and/or lyases cleave proteoglycans or GAGs during the inflammatory process, thus enhancing the exposure of WBC-EC adhesion molecules.

Figure 2.

Intravital microscopy of WBC-EC adhesion in post-capillary venules reveals a low level of adhesion under control conditions which increases dramatically upon exposure to chemoattractants (e.g. the peptide fMLP) [35]. A concurrent decrease in the thickness of the glycocalyx occurs. Inhibition of the effects of fMLP with the MMP inhibitor doxycycline mitigates the adhesion response and shedding of the glycocalyx [38].

Figure 3.

Fluid shear stresses (proportional to shear rate at the wall, $\dot{\gamma}$) cause WBCs to roll along the walls of small venules at velocity V_{WBC} . The strength of the adhesive interaction between WBC and EC is thus inversely proportional to the ratio $V_{WBC} / \dot{\gamma}$. Inhibition of MMP activity with either doxycycline (doxy) or the zinc chelator GM6001 mitigates the shedding of selectin receptors which increases the stickiness of WBCs to the EC. Similarly, fMLP causes shedding of the glycocalyx which exposes more ICAM-1 as well as increasing the avidity of integrin ligand-receptor binding which makes the EC sticky for WBCs and reduces rolling velocity. *Significantly less than control. Redrawn from [68].