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The Endothelial Glycocalyx as a Barrier to Leukocyte Adhesion and its Mediation by Extracellular Proteases

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Abstract

The endothelial cell (EC) surface is coated with a layer of polysaccharides linked to membrane bound and trans-membrane proteoglycans that comprise the glycocalyx, which is augmented by adsorbed proteins derived from the blood stream. This surface layer has been shown to affect hemodynamics in small blood vessels of the microcirculation, the resistance to flow and leukocyte (WBC) to EC adhesion. Parallel studies of WBC-EC adhesion in response to chemoattractants and cytokines, and shedding of constituents of the glycocalyx, have suggested a role for activation of extracellular proteases in mediating the dynamics of WBC adhesion in response to inflammatory and ischemic stimuli. A likely candidate among the many proteases present are the matrix metalloproteases, MMPs. Inhibition of MMP activation with sub-antimicrobial doses of doxycycline, or zinc chelators, have also inhibited WBC adhesion and shedding of glycans from the EC surface in response to the chemoattractant fMLP. Taken together, these studies suggest that shedding of the EC glycocalyx exposes adhesion receptors and thus enhances WBC-EC adhesion. Future therapeutic strategies for treating pathologies such as the low flow state and inflammation may benefit by further exploration of the mechanics of the glycocalyx in light of protease activation and shear dependent effects.

Historical Background

The interface between blood and endothelium has fascinated researchers for over a century and has received increased interest with the progression of advances in biochemistry and microscopy. Early studies on the structural makeup of the capillary wall by the eminent physiologist B. W. Zweifach drew attention to the surface of the endothelium as an essential part of the "hematoparenchymal barrier" [90]. It was recognized that endothelial cells continuously secrete substances which 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 that was first postulated by Danielli [15] to serve as a permeability barrier, and subsequently visualized by electron microscopy by Bennett and others [6; 54]. In view of its predominant polysaccharide constituents, Bennett [6] 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 of glycoproteins on the order of 50 to 100 nm thick, with a characteristic spacing of 20nm that accounts for the resistance to filtration of small molecules [76].

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Microvascular Hemodynamics

Interest in the role of the glycocalyx in affecting microvascular hemodynamics arose from the seminal studies of Klitzman and Duling [44] and Desjardin and Duling [17] in search 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 [64] and Fahraeus [20] noted reductions in capillary hematocrit that were well below 50% of systemic hematocrit [37; 67]. 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 [78]. Klitzman and Duling [44] hypothesized that the low capillary hematocrits arose from retardation of fluid at 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 [17] inserted finely drawn micropipettes into feeding vessels and perfused individual capillaries with heparinase to strip off the glycocalyx. Their results showed a two-fold 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 [9], or degradation due to the presence of reactive oxygen species derived from oxidized LDL [13].

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 [68]. 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) [65; 73]. Within small venules in the exteriorized cremaster muscle, these studies have revealed a glycocalyx thickness on the order of about 0.3 to 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 [73]. Thus, *in vitro* models clearly fail to replicate the *in vivo* structure of the glycocalyx.

Structure of the Glycocalyx

Studies of the dimensions and structure of the endothelial glycocalyx have been confounded by the methods of fixation and source of the cells studied [66; 72]. *In vivo* observations by direct microscopy have revealed an apparent thickness of the glycocalyx, estimated by the exclusion of erythrocytes and macromolecules [81], 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* [11]. 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.

Several studies to date have reviewed the structure of the endothelial glycocalyx [11; 25; 66; 72; 83]. 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 [72] and each PG may carry multiple chains of HS and CS, with a ratio of HS:CS of about 4:1 [70], and their sulfation level may change depending on the physiological micro-environment [70; 82]. 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 [46]. 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 [1; 38]. 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.

Shedding of the Glycocalyx

The labile nature of the glycocalyx has been demonstrated in models of the inflammatory process. 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 [33]. 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 [10]. Acute activation of the endothelium in post-capillary venules with the chemoattractant fMLP has been found to induce 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 [57]. Shedding of proteoglycans and GAGs from cultured endothelial cells, or their analogs, occurs in response to a broad spectrum of agonists [12; 21; 22; 40; 59–61]. Shedding of heparan sulfate proteoglycans (namely the ectodomain of syndecans 1–4) occurs in response to endotoxin [12], serine and/or cystein proteinases [39], complement activation [60], thrombin and growth factors [77] and activation of protein tyrosine kinase by phorbol ester [21]. 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 [21].

In vivo, the endothelial glycocalyx has been shown to be shed in response to inflammation [33; 57], hyperglycemia [89], endotoxemia and septic shock [34], presence of oxidized LDL [13], TNFα [10], atrial natriuretic peptide [7], abnormal blood shear stress [26; 31], ischemia-reperfusion injury [57], light induced production of free radicals [81] and during by-pass surgery [71; 79]. These observations have lead to an underlying connection between integrity of the glycocalyx and vascular homeostasis [57; 89]. Shedding of the glycocalyx in response to cytokines and chemoattractants occurs in all three principal divisions of the microvasculature: arterioles [33], capillaries [13; 33] and venules [33; 57]. With the majority of WBC adhesion receptors situated in post-capillary venules, as for example in the case of ICAM-1 [41], 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 suggests that several key enzymes, such as the matrix metalloproteases, may directly or indirectly be responsible for shedding of the glycocalyx components [58]. 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 [58]. The inhibitory activity of doxycycline on shedding results from its direct effect on MMP activation and not by its ability to chelate divalent cations [53], as evidenced by inhibition of MMP activation by the zinc chelating hydroxymic 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 [24]. However direct evidence that ROS cause shedding in response to chemoattractants or cytokines remains to be obtained.

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 activation of proteases and lyases synthesized by the endothelium [4; 57]. Increased synthesis of GAGs by cultured monolayers of ECs occurs with prolonged exposure to high shear stresses of 15 or 40 dyn/cm^2 [4]. 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 [27]. *In vivo* studies of the accumulation of glycans on the surface of post-capillary venules during a one hour period of ischemia demonstrated a 15–40% increase in glycan content on the surface of the EC [57]. 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 post-ischemic 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.

Extracellular Proteases

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 [74]. MMPs (−1 and −9) serve to cleave the endothelial insulin receptor and CD18 on leukocytes in the spontaneously hypertensive rat [16]. Oxidative stress in the diabetic heart may activate MMP-2 and lead to the development of diabetic cardiomyopathy [85]. Modification of the extracellular matrix by MMPs has been shown to be a critical step in angiogenesis [29] and atherosclerosis [52]. MMP-2, MMP-7 and MMP-9 were shown to be capable of directly cleaving chondroitin sulfate [28]. In addition, MMP-1 was shown to cleave the heparan sulfate proteoglycan syndecan-1 [19]. MMPs can be stored within and released by the endothelium. It has been shown [80] 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 [86]. 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 [86].

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 [58]. 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) [19], shedding of syndecan −1 and −4 from HeLa tumor cells by MMP-9 [8], shedding of syndecan-1 from pancreatic carcinoma cells by MMP-7 [18], and shedding of syndecan-1 by MMP-7 during transmigration of neutrophils from the interstitium to alveoli in the lung [51].

Leukocyte Rolling and Adhesion

Based upon the rolling and adhesion of WBCs on either artificial surfaces coated with receptors for specific ligands [2; 48; 49], or monolayers of cultured endothelial cells [4; 35; 47], it has long been held that adhesiveness was governed by regulation of the affinity and avidity of the integrin molecules on the WBC and EC [43; 45; 55; 87]. *In vivo* studies of post-capillary venules in the living animal [3; 36; 50] 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. As reviewed recently, WBC microvilli may range in length from 0.3 to 0.7 μm [83]. Thus, the ability to penetrate the glycocalyx may depend on changes in porosity and stiffness attendant to physiological stimuli [62; 63; 83]. 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 [75]. Thus, given that the apparent thickness of the glycocalyx (typically 400–500 nm [81]) may be significantly reduced by either the chemoattractant fMLP [23] or the cytokine TNF-α [33], it appears logical to consider shedding as a promoter of WBC-EC adhesion. Perfusion of post-capillary venules with heparinase or superfusion of the tissue with fMLP served to increase exposure of ICAM-1 on the EC surface [56]. Thus, it has been postulated that stimulated shedding of the glycocalyx may rapidly expose receptors on the EC to facilitate WBC-EC adhesion [57]. 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 be relatively more selective for heparan sulfate) produced an increase in firm WBC adhesion [14]. Alternatively, it has been demonstrated that heparinase reduces stimulated rolling and adhesion of WBCs in post-capillary venules by affecting the externalization of P-selectin and/or compromising the structural interactions between heparan sulfate proteoglycans and selectins [32].

The concurrent events of shedding of glycans from the EC and enhanced adhesion of WBCs supports the hypothesis that modification of the endothelial glycocalyx by protease activity at its surface may rapidly affect the rolling and firm adhesion of WBCs. The interplay between WBC rolling and adhesion during an inflammatory stimulus (fMLP) is summarized in Fig. 2, based upon previous data [53]. Following activation of both WBCs and ECs with fMLP, firm adhesion quickly ensues. This adhesion can be effectively eliminated by addition of the MMP inhibitor doxycycline. Shedding of glycans from the EC surface quickly follows, as evidenced by a reduction in lectin coated fluorescent microspheres bound to the EC. This shedding is also inhibited by suppression of MMP activity with doxycycline. 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}/S.R. the greater the adhesiveness) it is apparent that superfusion of the tissue with EDTA disrupts adhesive bonds, whereas doxycycline enhances them. A similar decrease in V_{WBC}/S.R. results from the MMP inhibitor GM6001. These data 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 [58]. 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 exposure of adhesion receptors due to externalization of receptors and shedding of the glycocalyx. While

inhibition of MMP activation and fMLP both result in diminished rolling velocity, the strength of the adhesive bond during firm adhesion remains to be evaluated. Preliminary data to date suggest that the adhesive bond formed between WBC and EC in mesenteric venules is much weaker in response to MMP suppression, compared to that with fMLP, presumably due to conformational changes of integrin receptors induced by fMLP.

These events 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 [30], 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 [84]. Thus, if this endothelial selectivity of the MMP inhibitors GM6001 and doxycycline applies to the reductions of WBC rolling velocity in venules (as summarized in Fig. 2), 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 [42]. 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 [33], and the enhanced exposure of adhesion ligands (e.g. ICAM-1).

Directions for Future Study

In summary, substantial evidence suggests that the glycocalyx on the luminal surface of vessels within the microvasculature is a labile structure whose composition may be rapidly affected by metabolic and inflammatory events. It is a barrier between blood cells and endothelium that may be rapidly modified by extracellular proteases to affect microvascular hemodynamics and blood cell adhesion. MMPs may be the source of shedding of glycans in response to endothelial activation by cytokines and chemoattractants and further studies are needed to elucidate the specific proteases and their inhibitors involved. The identification of the specific protease responsible for shedding may involve numerous complexities due to the ability of specific MMPs to activate other MMPs as well as other families of proteases, and for other proteases to activate MMPs. For example, there is an indirect association between heparanase and MMP expression [69; 88]. Blocking activation of MMP-9 inhibited heparanase induced syndecan-1 shedding in myeloma cells [69] and over expression of heparanase in cultured human mammary carcinoma cells resulted in diminished expression of MMP-2, -9 and 14 [88]. Future studies on the role of fluid shear stresses on the structure and deformation of the glycocalyx are also warranted. Red cell adhesion in sickle cell disease [5] and WBCs in inflammation [36] are shear dependent phenomena. The role of deformation of the glycocalyx in affecting the adhesive bond remains to be explored. Coupling studies of the mechanics of cell adhesion and deformation of the glycocalyx might lead to new insights to strategies for treating pathologies such as the low flow state and other disorders.

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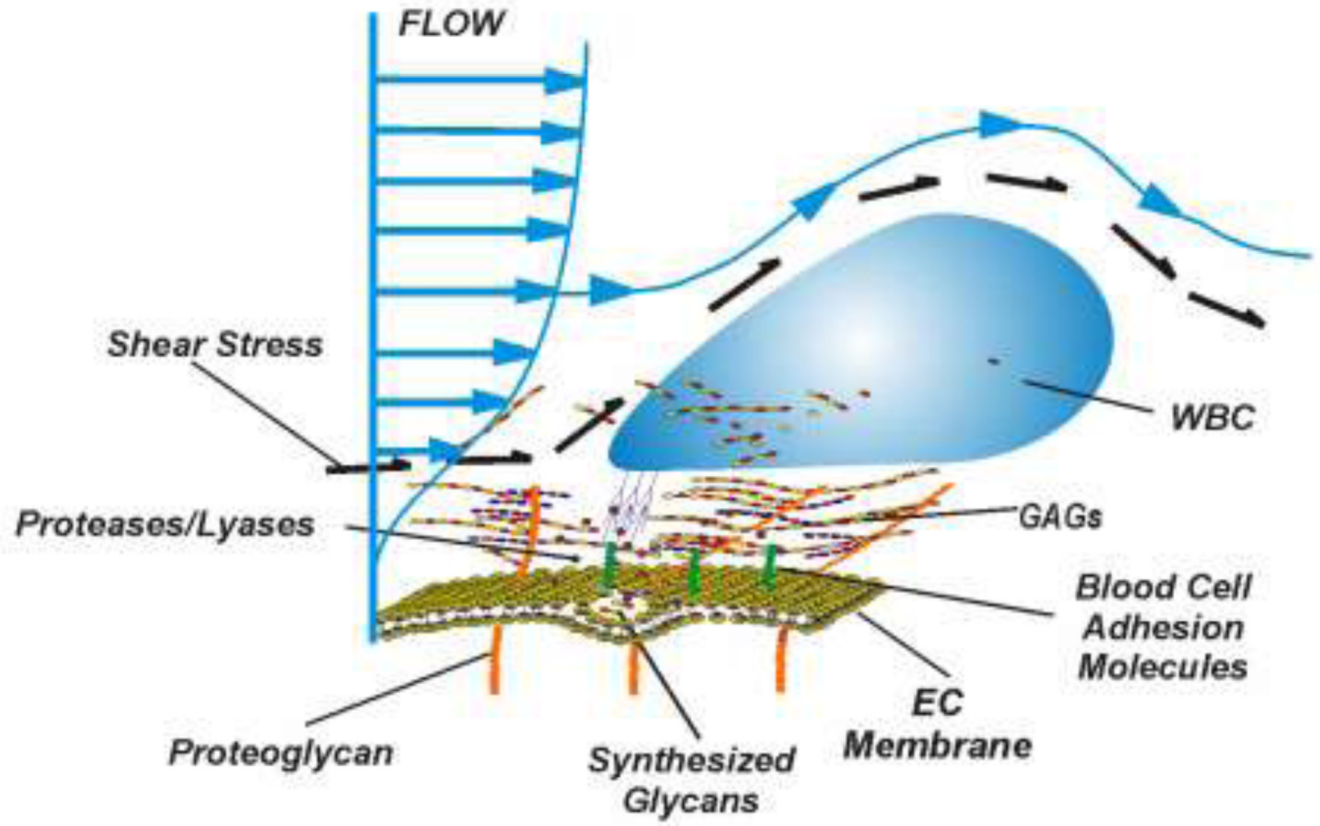

Figure 1.
Hypothetical role of the endothelial glycocalyx on leukocyte-endothelium adhesion. Adhesion receptors on the endothelium are buried deep within the glycocalyx. Activation and/or externalization of proteases or lyases may degrade the glycocalyx by cleavage of proteoglycans and/or glycosaminoglycans (GAGs). Shedding of the glycocalyx may thus facilitate ligand-receptor interactions that promote firm adhesion of WBCs. Basal levels of activated proteases causes a continuous shedding of glycosylated proteins that result in a continuous shedding of substrates that support WBC rolling. Inhibition of sheddase activity causes the EC surface to become more sticky and retard the rolling motion of WBCs.

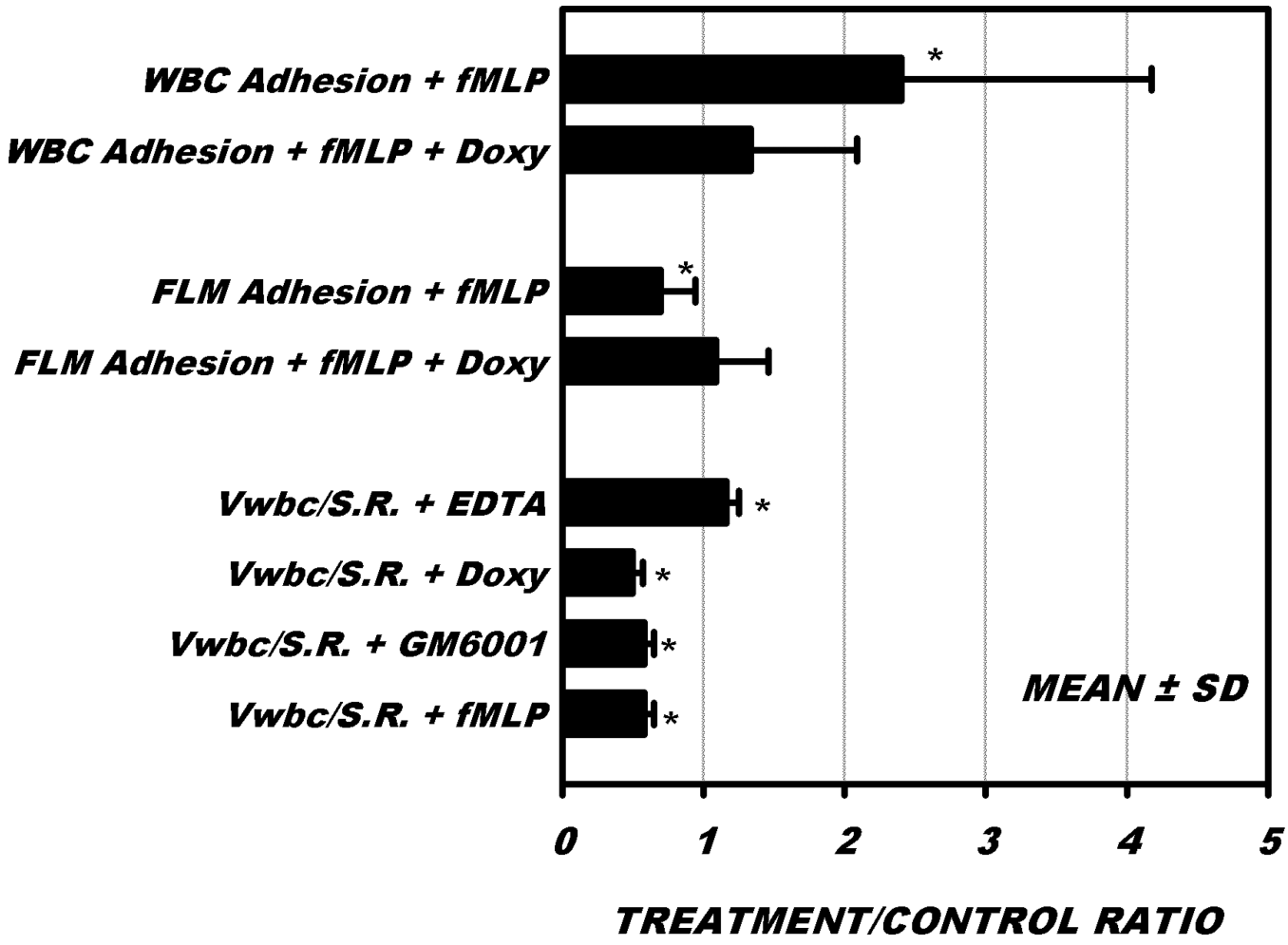

Figure 2.

Leukocyte (WBC) rolling velocity and adhesion, and adhesion of lectin (BS-1) coated fluorescently labeled microspheres (FLMs) in post-capillary venules following superfusion of mesentery with either the chemoattractant fMLP (10^{-7} M), with and without addition of the matrix metalloprotease inhibitors doxycycline (0.5 μM) or GM6001 (2.5 μM). Also shown is the rolling velocity in response to 3 mM EDTA. From top to bottom: A significant rise in WBC-EC adhesion occurs in response to fMLP, which is mitigated by the addition of doxycycline. A significant fall in lectin bound FLMs signifies shedding of glycans from the EC in response to fMLP, which is also mitigated by doxycycline. Rolling velocity of WBCs increases significantly with EDTA, as chelation of divalent cations diminish the avidity of adhesion receptors that govern rolling. Significant reductions in rolling velocity occur as the number of adhesion receptors are increased with inhibition of basal sheddase activity by doxycycline or GM6001. These results are normalized with respect to prevailing wall shear rates (S.R.). Superfusion of fMLP also causes a significant reduction in rolling velocity, consistent with exposure of more receptors as glycans are shed from the EC. *Significantly different from control value.