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Dual Regulation of L-Selectin-Mediated Leukocyte Adhesion by Endothelial Surface Glycocalyx

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Abstract—Endothelial surface glycocalyx (ESG) is a carbohydrate-rich, gel-like layer found on vascular endothelium, serving critical functions in mechanotransduction of blood flows, maintenance of the endothelial permeability, and the control of leukocyte adhesion and inflammation. This study aimed to clarify the role of ESG in the adhesion between leukocytes and Human Umbilical Vein Endothelial Cells (HUVECs) under resting or inflammatory conditions. Using an atomic force microscopy-based single-cell adhesion assay, we directly quantified the detachment force and work perpendicular to the cell membrane. Detachment force and work were measured for every separation event of a leukocyte from a HUVEC with ESG, or with the major ESG glycosaminoglycan components, heparan sulfate (HS) and hyaluronic acid (HA) removed. For the resting HUVECs, when HS and/or HA were removed, the detachment force and work increased dramatically. For the HUVECs activated by inflammatory cytokine tumor necrosis factor alpha, we observed increases in the detachment force and work compared to the resting HUVECs, and removal of HS and/or HA resulted in significant decreases in the detachment force and work. The results demonstrate that the ESG layer serves a dual function: (1) on resting endothelium, it prevents leukocyte adhesion, and (2) under inflammatory conditions, it participates in endothelial-leukocyte interactions with molecules such as selectins.

Keywords—Atomic force microscopy, Endothelial surface glycocalyx, Heparan sulfate, Hyaluronic acid, K562 cells, Human umbilical vein endothelial cells, Tumor necrosis factor alpha, L-selectin.

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INTRODUCTION

The endothelial surface glycocalyx (ESG) exists on the luminal surface of the entire vasculature and is constantly being exposed to cell–cell interactions. ESG is a negatively charged layer composed mainly of glycosaminoglycan (GAG), proteoglycans and glycoproteins.[11,36] The net negative charge of the ESG is due mainly to the major GAG components, heparan sulfate (HS), hyaluronic acid (HA) and chondroitin sulfate (CS).[11] The ESG is thought to capture circulating plasma protein and to form an organized gel-like structure in water-based solvent environments.[33,43,49]

Among other important functions, the ESG is known to act as a buffer to cell–cell adhesion between leukocytes, platelets and other circulating blood cells to the endothelial wall.[7,12] Furthermore, activation of adhesion molecules, such as selectins, is widely believed to change the properties of the ESG[38,42] and to cause it to lose its non-adhesive properties.[7,12] The same loss of non-adhesive ability is observed when the glycocalyx is exposed to digestive enzymes, such as heparitinase or heparanase.[7,22]

GAGs, HS, HA and CS are linear polymers of disaccharides that have varying lengths and are modified by sulfation.[36] HS comprises approximately 50–80% of the total GAGs[15,35] and is typically found in a 4:1 ratio with CS.[28] These GAGs form chain-like structures that are held to the surface of endothelial cells by proteoglycans and glycoproteins. Proteoglycans consist primarily of syndecans, glypicans and glycoproteins, such as CD44, and form the backbone of the ESG. Syndecans-1, -2, -4 contain attachment sites for HS, and syndecan-1 also harbors two more attachment sites for CS near the cell membrane.[11] Glypican-1, the only known glypican to be expressed

on the surface of endothelial cells, is anchored to the cell surface through a glycosylphosphatidylinositol (GPI) anchor[10] and interacts exclusively with HS.[12] The transmembrane glycoprotein CD44 is the only binding site for HA[12,29] and also contains binding sites for CS.[12]

Interactions between the vascular endothelium and circulating leukocytes represent not only a crucial event in immune surveillance and defense but are also critically involved in the pathogenesis of many inflammatory and immune diseases. Although it has been known for decades that the ESG serves as critical functions in regulating leukocyte adhesion and inflammation, the exact role(s) played by ESG in mediating leukocyte-endothelial interaction has yet to be clarified. In this study, we quantified, *via* atomic force microscopy (AFM), the separation forces that exist between a leukocyte and an endothelial cell, imitating the adhesion force of the first tethering and rolling stage of leukocyte adhesion. We report that an intact ESG layer is necessary to prevent leukocyte adhesion on resting endothelium; and that under inflammatory conditions, an intact ESG participates in endothelial-leukocyte interactions with molecules such as selectins.

MATERIALS AND METHODS

Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVECs), purchased from ATCC (Ref. PCS-100-010), were cultured in Lonza EBM-2 medium from an EGM-2 BulletKit (Ref. CC-3162). K562 leukocytes, purchased from Sigma-Aldrich (Ref. 89121407), were cultured in RMPI-1640 medium also from Sigma-Aldrich (Ref. R0883) containing 10% FBS from ATCC (Ref. 30-2020), 2% L-glutamine from Gibco by Life Technologies (Ref. 25030081), 1% sodium bicarbonate (Ref. S8761) from Sigma-Aldrich, 1% from sodium pyruvate from Sigma-Aldrich (Ref. S8636) and 1% penicillin–streptomycin from Sigma-Aldrich (Ref. P4333).

All cells were cultured in Corning T-25 flasks and plated in 35 mm Cyto-One treated culture dishes for AFM experiments or in World Precision Instruments 35 mm FluoroDish glass bottom culture dishes for cell staining. The cultures were maintained at 37 °C and 5% CO_2 in a water jacketed incubator.

Heparinase I&III blend from flavobacterium heparinum (Ref. H3917), Hyaluronidase from bovine testes (Ref. H3506), wheat germ agglutinin (WGA) (Ref. L4895), Poly-L-lysine from Sigma-Aldrich (Ref. P8920), and Collagen type I solution from rat tail (Ref. C3867-1VL) were purchased from Sigma-Aldrich. TNF-α (Ref. 210-TA) was from R&D systems (Minneapolis, MN). DREG.200 (Ref. HB-302) was from ATCC. Anti-MYC (Ref. R950-25) was from Invitrogen. Alexa Fluor 546 goat anti-mouse secondary anti-body (Ref. A-11003) and Hoechst 33258 pentahydrate (bis-benzimide) (Ref. H21491) were from ThermoFisher.

HUVEC Sample Preparation

The HUVECs were seeded on a 22x22-1 glass cover slip (Fisher Scientific Ref. S175211), placed inside a 35-mm culture dish, 24 h prior to the experiments. The glass cover slip was pre-treated with of 0.2 mg/mL collagen to achieve optimum cell growth. For the AFM adhesion assay, 15% of culture confluence was reached to insure that single HUVECs were present and to ensure that the K562s that were being picked up by the AFM were not in contact with any HUVECs prior to the experiment.

For HUVEC activation experiments, the HUVECs were stimulated with TNF-α for 10 h at 37 °C at a concentration of 100 ng/mL. The heparinase I&III treatment and/or hyaluronidase treatment were performed immediately before the experiment and were performed at concentrations of 25 mU/mL and 20 μg/mL, respectively. First, the cells were treated with the proper concentrations of heparinase I&III and/or hyaluronidase and placed in the incubator for 1 h. After removal from the incubator, the cells were washed 3 × with the culture medium to remove any remaining enzymes, cytokines and cleaved ESG constituents. If the cells were stimulated with TNF-α, the procedure was performed prior to heparinase I&III treatment and/or hyaluronidase treatment. There was no washing procedure performed in between treatments.

AFM Probe Preparation

The AFM probes used in this study were MLCT-O10 tipless nitride levers and were purchased from Bruker Nano. To begin the experiment, the AFM probes were first coated with poly-L-lysine *via* a high pH buffer technique. First, the AFM probes were placed in acetone for 10 min, and then they were sterilized in a UV ozone chamber for 20 min. After removal from the chamber, they were placed into a 0.2 M solution of $NaHCO_3$ in DI water (pH 9.0) containing a 50 μg/mL concentration of poly-L-lysine. The probes were left to incubate in this solution over night at 4 °C.

AFM Instrument

The AFM used in all experiments was a custom built machine that was programmed using IGOR. The AFM was attached to a CCD camera with a × 20

objective, suspended from the ceiling *via* elastic cables and housed in a noise damping container. The spatial resolution of the AFM in the x-direction is approximately 1 nm, and the spatial resolution in the y-direction is approximately 15–20 pN.

AFM calibration was performed first by pressing the probe against the surface of a Cyto-One culture dish to determine the inverse optical lever sensitivity (InvOLS). The InvOLS was defined as a measure of the sensitivity of the photodetector for the motion of the laser as it reflected off of the deflecting probe. This value was determined by the slope of the contact portion of the trace produced by the AFM as the probe touched the surface of the dish. Next, the AFM probe was vertically moved at least 2 mm away from the surface of the culture dish and the random thermal fluctuations of the AFM probe were recorded as a function of time such to determine the spring constant using an established method by Hutter *et al.*[14] With the InvOLS in m/V and the spring constant in N/m, a change in voltage at the photodetector could then be converted into a force at the probe. The poly-L-lysine coating caused some adhesion between the probe and the surface of the dish; however, there was no deviation from the expected InvOLS and spring constant values. PBS was utilized as the fluid during the calibration procedure.

AFM Single-cell Adhesion Assay

The assay was slightly modified from previous work done by us and others.[21,52] To begin the assay, the K562 cells were spun down, re-suspended in EBM-2, and then added to the HUVECs. For the experiments using the TNF-α-activated HUVECs, the K562s were not activated by TNF-α, as this may cause L-selecting shedding.[17] The K562s were allowed to sit at room temperature for 15 min, under the AFM, before the experiment began, such that the AFM probe would reach thermo-equilibrium with its surrounding fluid and so that the leukocytes would have time to settle on the bottom of the dish.

To perform the experiment, a live K562 cell was first attached to the end of the AFM probe by lowering the probe down and touching it to the cell, which sat unadhered to the surface of the culture dish. Because the K562 cells possess net negative charges and the cantilever possesses a net positive charge due to the poly-L-lysine, the cells were easily picked up from the bottom of the dish; the force used to capture the leukocyte was 1 nN with 5 s of dwell time.

Immediately after capturing the cell, the adhered K562 cell was brought over the top of an HUVEC, away from the nucleus, and the force scanning began. The two cells were touched together with a force of approximately 300 pN with a dwell time of 0.5 s resulting in a contact area of approximately 17.35 μm^2. The contact area was calculated from a standard contact mechanics model for elastic spheres[53] (Fig. S1). More information on the determination of this value can be found in the supplemental material. In all experiments, the AFM recorded the force of indentation, the interaction between the two cells and the extension of the probe tip for 30 force scans per cell–cell pair. All of the data were analyzed for peak force and work (Fig. 1). Unique cell–cell pairs were chosen each time, i.e., a new HUVEC and a new K562 were selected every time.

Immunostaining and Fluorescence Microscopy

To verify the presence of L-selectin, an antibody assay was conducted. To begin, a Precision Instruments 35 mm FluoroDish glass bottom culture dish was coated with poly-L-lysine by diluting poly-L-lysine in a 0.2 M solution of $NaHCO_3$ in DI water (pH 9.0) containing a 200 μg/mL concentration of poly-L-lysine. The dishes were left to incubate in this solution overnight at 4 °C. They were then washed three times with PBS, and the cells were added to the dishes in their culture medium. After 1 h, the culture medium was removed, and the cells, now immobilized on the bottom of the dish, were fixed with 4% W/V paraformaldehyde for 10 min. They were then washed twice for 10 min in PBS. Next, DREG.200 was added to the PBS at a concentration of 10 μg/mL, and the cells were allowed to sit overnight at 4 °C. After this, the cells were treated with the secondary antibody Alexa Fluor 546 goat anti-mouse at a concentration of 10 μg/mL for one hour and the nucleus stain Hoechst 33258 pentahydrate at a concentration of 20 μg/mL for 5 min. Finally, the cells were washed twice with PBS and imaged using a Nikon C2si confocal microscope in PBS. Controls were performed with both 10 μg/mL of anti-MYC and no primary antibody.

WGA Staining and Fluorescence Microscopy

WGA, which in this case is an FITC conjugate, has the ability to recognize and bind to *N*-acetylglucosamine,[20] a common disaccharide unit within HS and HA.[1,37,39,40] The cells were then washed twice with PBS and treated with 5 μg/mL of WGA for 10 min at 37 °C in PBS supplemented with 5% BSA. The cells were then washed two additional times in PBS and imaged using a Nikon C2si confocal microscope in PBS with 5% BSA. For the GAG removal experiments, prior to WGA staining, HUVECs were treated with heparinase I&III at a concentration of 25 mU/

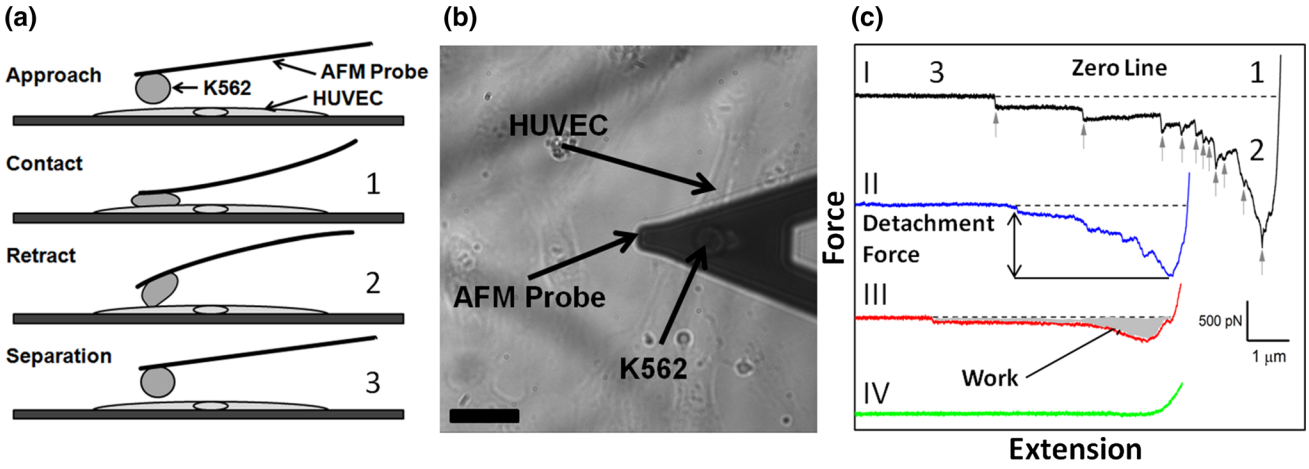

FIGURE 1. An AFM assay of leukocyte-endothelial interaction. (a) A schematic of the experimental procedure. (b) Image of a single leukocyte adhered to the AFM probe over a single HUVEC. Scale bar represents 20 μm. (c) Typical force spectrum traces for K562 cells bound to HUVECs under different conditions. Strong adhesion occurred between K562s and HUVECs treated with TNF-α (trace I). Arrows in the black trace indicate rupture events, i.e., breakage of adhesive bond(s). Dashed lines indicate zero forces. The adhesion between K562 cells bound to HUVECs after TNF-α by was markedly reduced by hyaluronidase treatment (trace II). The "detachment force", which is the peak force required to separate the bound K562s from the HUVECs, is labeled for trace II. This was again observed under heparanase I&III treatment (trace III). The shaded area in the trace III is the "work of de-adhesion." On resting HUVECs (trace IV), adhesion (both detachment force and work) was minimal. Measurements were acquired with a compression force of 300 pN, a 0.5 s contact, and a cantilever retraction speed of 3 μm/s.

mL, and/or 20 μg/mL hyaluronidase for 1 h 30 min at 37 °C in the culture medium.

Statistical Analysis

All statistical analyses were carried out using IBM SPSS Statistics 19 (SPSS, Inc., Chicago, Ill., USA). Data normality was verified by Shapiro–Wilk testing and by checking the Q–Q plots. If normality was met, statistically significant differences between treatment groups were identified using ANOVA with least significant difference (LSD) *post hoc* testing. If normality was not met, Kruskal–Wallis H tests were performed, and pairwise comparisons were made using a Bonferroni correction. The results of these tests (p values) are presented in each figure, and a detailed description of the statistical analysis for each group can be found in the supplemental information.

RESULTS

In this study we used AFM to quantify the adhesion between leukocytes and the endothelial cells. We elected to use K562 cells as a model system of human leukocytes. K562s are a leukocyte cell line that can impulsively develop characteristics that are analogues to erythrocytes, granulocytes and monocytes.[24] Moreover, K562s are not known to express, among other adhesion molecules, endogenous L-selectin.[5] L-selectin, belongs to a group of lectins known as selectins that arbitrate the initial adhesion of leukocytes to the luminal surface of the endothelium in a Ca^{2+} dependent fashion during inflammation.[41,44] K562s have been widely used in model systems to study leukocyte adhesion.[3,5,6,13,26]

The first aim of the study was to identify the role of ESG in regulating the interaction between K562 and resting HUVECs. As shown in Fig. 2, K562 adhesion on resting HUVECs are minimal, with averaged detachment force and work being 81 pN and 91 aJ, respectively. A detachment force of 50 pN has been reported typically for weak, non-specific interactions (ref). When the HUVECs were exposed to heparanase I & II and/or hyaluronidase, the work of de-adhesion and peak force required to separate the cells increased significantly by about 5 and 2 fold, respectively, indicating that the GAG components of ESG, HS and HA may play a negative regulatory role in K562-HUVEC adhesion.

The second aim of this study was to examine the role of ESG in leukocyte-endothelial adhesion under inflammatory conditions. In order to mimic inflammatory conditions, we treated the HUVECs with TNF-α. TNF-α is an inflammatory cytokine produced by macrophages and monocytes during acute inflammation and is responsible for a varying range of signaling events within cells, leading to necrosis or apoptosis.[16] Additionally, the expression of adhesion molecules L-, P-selectin is up-regulated *via* TNF-α,[37] and TNF-α has been shown to up-regulate monocyte and leukocyte attachment through L-selectin

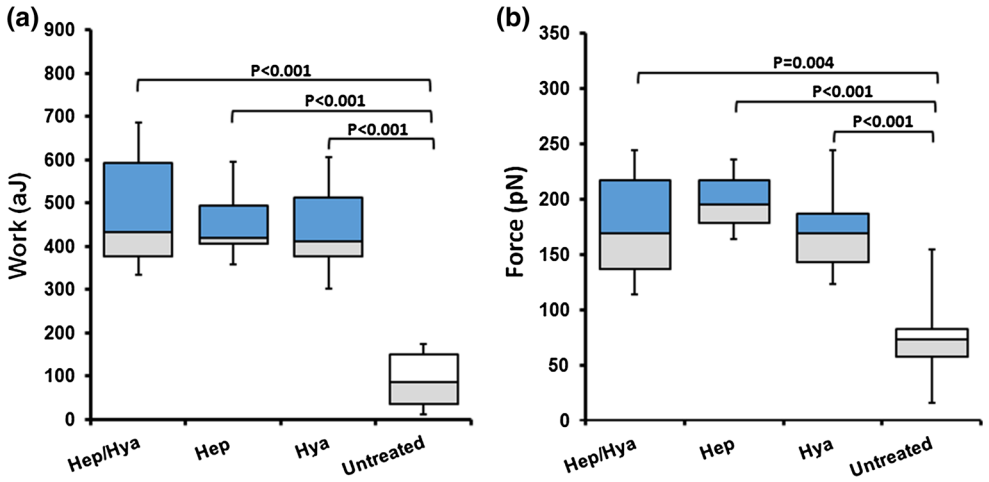

FIGURE 2. ESG-mediated adhesion on resting HUVECs. (a) Work of de-adhesion involved in rupturing all bonds and (b) detachment force between the K562s and the HUVECs. The *P* values indicate the statistical differences between the indicated groups. N = 10 cell–cell pairs for GAG digested, and N = 15 cell–cell pairs for resting HUVECs. Heparinases I&III and hyaluronidase are abbreviated as Hep. And Hya., respectively. The error bars for the box and whiskers plots were determined by the maximum and minimum values of the data sets.

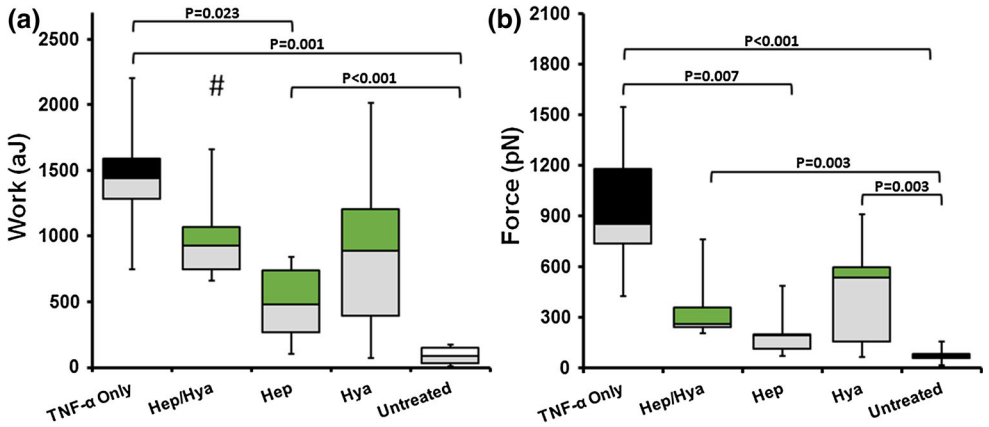

FIGURE 3. ESG-mediated adhesion on TNF-α-activated HUVECs. (a) Work of de-adhesion involved in rupturing all bonds between the K562s and the HUVECs. (b) The deadhesion force required to separate the bound K562s from the HUVECs. The p values denote the statistical differences between the indicated groups. N = 10 cell–cell pairs for the TNF-α alone, heparanase and hep/hya groups, N = 13 cell–cell pairs for hyaluronidase group, and N = 15 cell–cell pairs in resting HUVECs group. Heparinases I&III and hyaluronidase are abbreviated as Hep. And Hya., respectively. The error bars for the box and whiskers plots were determined by the maximum and minimum values of the data sets. Outliers are designated by hash.

binding.[12,50] TNF-α is often used in *in vitro* model to resemble an inflamed endothelium.

Shown in Fig. 3, upon TNF-α stimulation, the K562-HUVEC adhesion was greatly increased, yielded an 11.5 fold increase in detachment force and 15 fold increase of detachment work compared to resting HUVEC. Adhesion between K562s bound to activated HUVECs was dramatically decreased by heparanase I&III treatment, indicating that the increase in interactions was almost entirely dependent upon the presence of heparin groups during TNF-α stimulation (Fig. 3). Curiously, the same affect, was not observed in significant fashion under hyaluronidase treatment of the endothelial cells.

To verify the removal of the GAGs from the surface, a fluorescence staining assay using WGA-FITC was performed (Fig. 4). The images clearly showed a reduction in WGA fluorescence in the HUVECs treated with heparanase I&III. The same procedure was performed for the cells after hyaluronidase treatment. Interestingly, only a very small difference in fluorescent intensity could be detected, as shown in Fig. 2. This may be because HA makes up a much smaller portion of the total GAGs than HS. Because WGA is specific

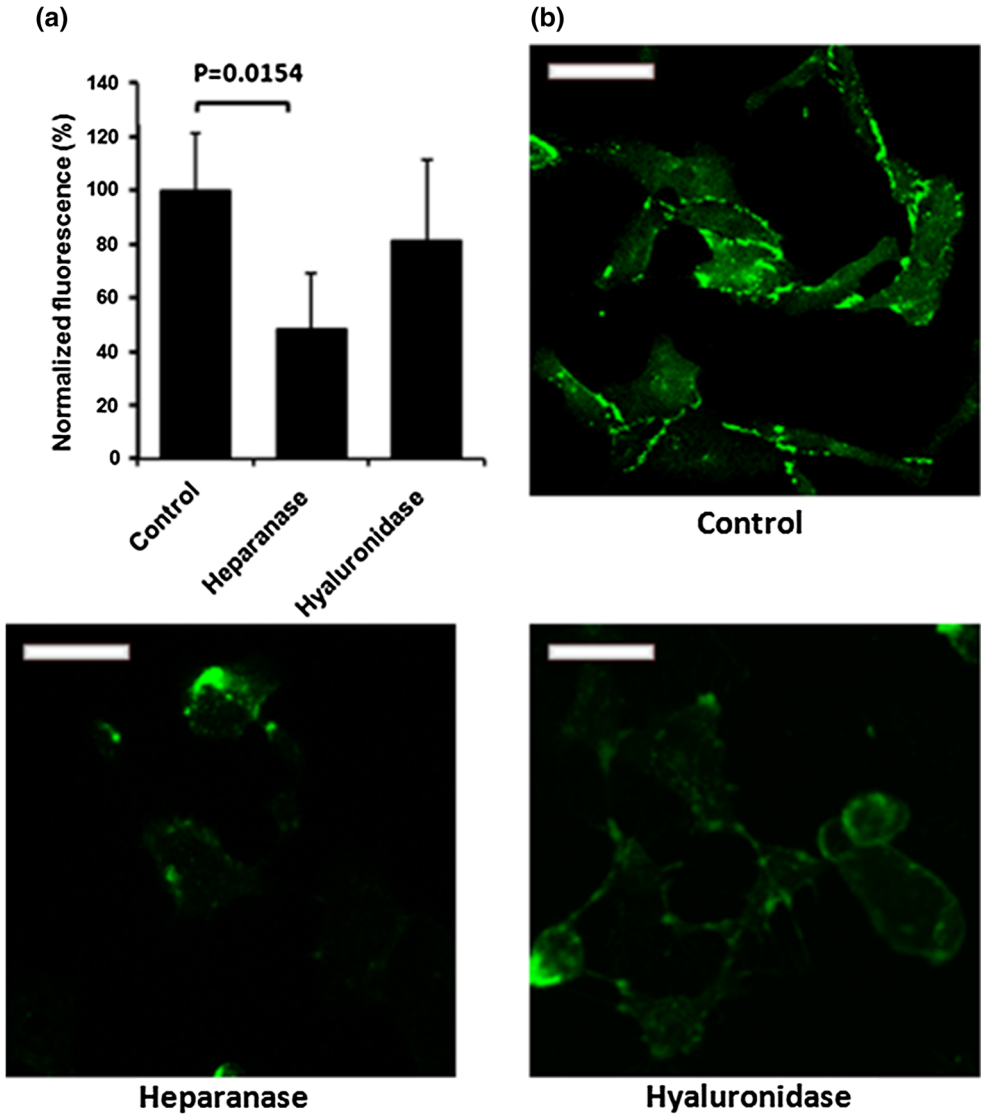

FIGURE 4. Fluorescence images of GAG. (a) Plot of the normalized WGA-FITC fluorescence intensity of untreated and GAG-digested groups. (b–d) are representative images of the three groups. Scale bar represents 20 μm.

only to *N*-acetylglucosamine, a disaccharide unit that HS and HA have in common, it is reasonable to believe that the background on the cell surface was too high to detect a significant difference in fluorescence if HA alone is removed.

Next, we sought to identify the mechanism underlying the enhanced K562 adhesion upon HUVEC activation. L-selectin is typically expressed on leukocytes and interacts with constitutively expressed ligands and inducible ligands on the endothelium.[23,41] L-selectin tethering activity was blocked using the mouse derived IgG1 antibody DREG.200, which is known to block the functional groups of L-selectin.[18,25,46,48] Figure 5a, 5b demonstrate remarkable decreases in the force and work after the leukocytes were exposed to DREG.200 for 15 min following 10 h of TNF-α stimulation of the HUVECs. A control was conducted with the mouse IgG antibody anti-MYC (non-L-selectin blocking) after 10 h of TNF-α stimulation of the HUVECs. The results the anti-MYC control showed little difference in adhesion strength compared to the TNF-α only group (Fig. 5a, 5b).

Moreover, the presence of L-selectin on K562s was verified *via* antibody immune-staining (Fig. 5c, 5d). The results showed a clear expression of L-selectin on the surfaces of the K562s used in this experiment, which demonstrates that these K562s do express L-selectin functional groups. Since L-selectin-mediated adhesion is dependent on Ca^{2+}, to further confirm the involvement of L-selectin in K562-HUVEC interaction, calcium chelation experimentation was also performed on resting HUVECs and HUVECs stimulated with

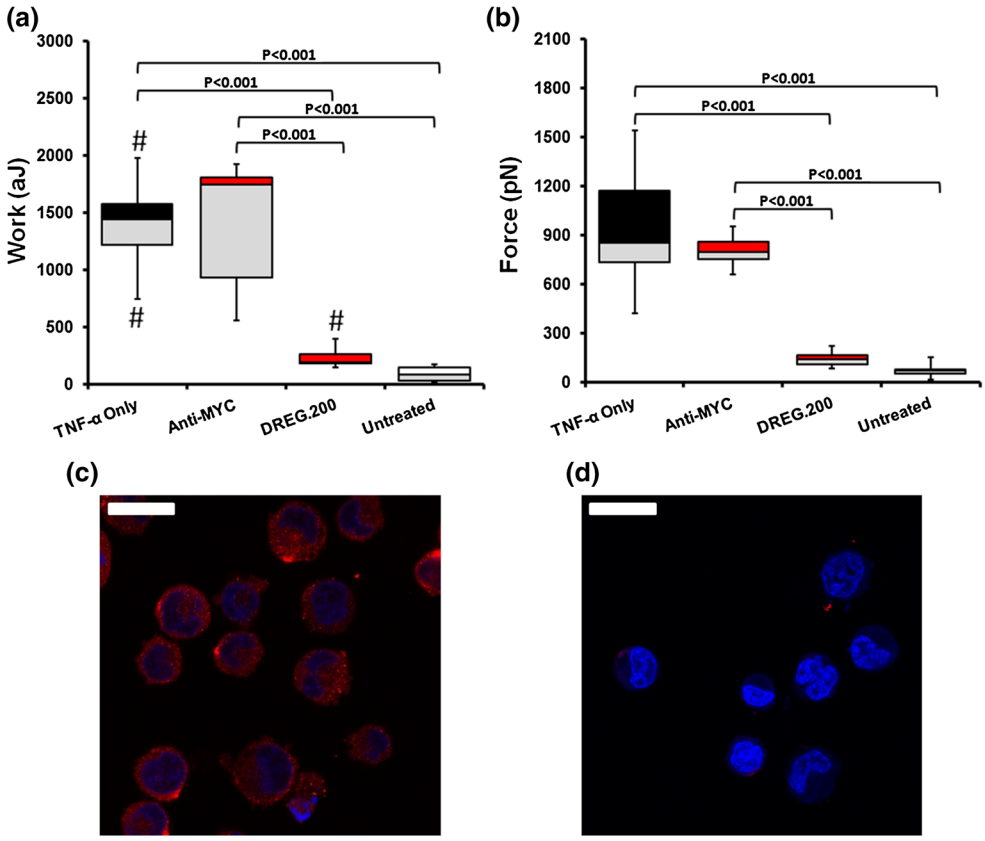

FIGURE 5. TNF-α-induced adhesion is mediated by L-selectin. (a) The work of de-adhesion involved in rupturing all bonds between the K562s and the HUVECs. (b) The peak force required to separate the bound K562s from the HUVECs. N = 15 cell–cell pairs for the resting HUVEC group. N = 10 cell–cell pairs for all other experimental conditions. The error bars for the box and whiskers plots were determined by the maximum and minimum values of the data sets. Outliers are designated by *hash*. Representative immuno-staining images of the K562s stained for DREG.200 and detected Alexa Fluor 546 goat anti-mouse secondary antibody (c), and K562s treated with anti-MYC 24 and detected by secondary antibody (d). In all images, the nuclei are stained with Hoechst 33258, shown in blue.

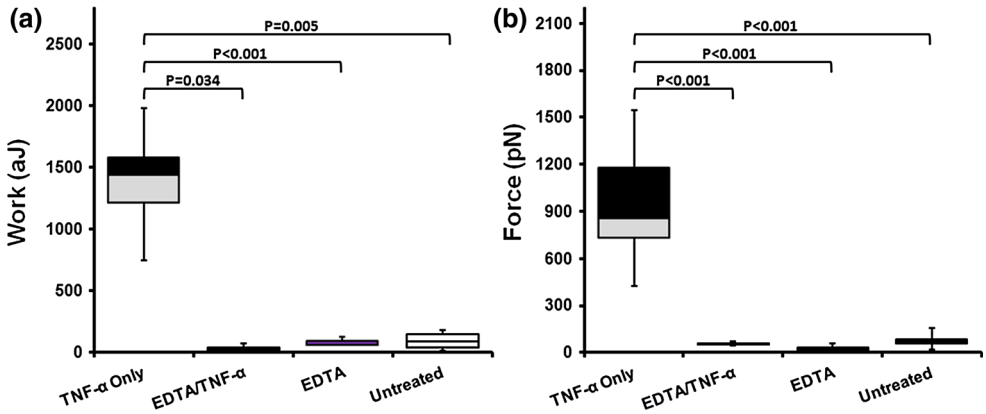

FIGURE 6. EDTA blockage of TNF-α-induced adhesion. (a) Work of de-adhesion involved in rupturing all bonds between the K562s and the HUVECs. (b) The peak force required to separate the bound K562s from the HUVECs. The p values denote statistical differences between the indicated groups. N = 15 cell–cell pairs for the resting HUVEC group. N = 10 cell–cell pairs for all other experimental conditions. The error bars for the box and whiskers plots were determined by the maximum and minimum values of the data sets.

TNF-α (Fig. 6). The reaction molecule Ca^{2+} was removed from the culture medium *via* 10 mM EDTA, which is known to remove free Ca^{2+}. EDTA was added directly to the dish 10 min before the experiment began. Removing Ca^{2+} from the medium caused a reduction in the unbinding force and work for both the

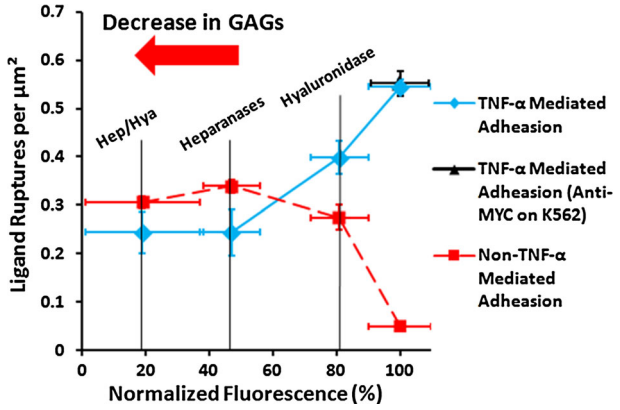

FIGURE 7. Ligand ruptures between the K562 and HUVEC. The ligand ruptures per μm^2 quantified as a function of GAGs present on the cell surface, as determined by the WGA FITC fluorescence. The –control, TNF-α (DREG.200 on K562), EDTA, and TNF-α EDTA were excluded from this plot, as there was <0.05 per μm^2 of rupture on average for these conditions. The vertical lines represent the types of enzymatic digestions.

resting and TNF-α-stimulated HUVECs (Fig. 6). This information demonstrates two possibilities (1): that L-selectin is dependent on Ca^{2+} and therefore L-selectin binding is inhibited, or (2) other long chain molecules, such as Lewis^x, are also in part responsible for the interactions.

DISCUSSION

The ESG is a negatively charged[11,36,51] gel-like structure[33,43,49] that is predominately made up of HS [15,35] and can be approximately 0.5 μm in height.[32,46,47] Leukocytes also have a thin, negatively charged glycocalyx with an effective thickness of approximate 15 nm.[2] Therefore, on resting cells, the ESG may down-regulate leukocyte adhesion *via* electrostatic repulsion. When the HUVECs were exposed to heparinase I&III, the thickness, density and charge of ESG may have been reduced. This could allow for leukocyte adhesion molecules that localize on the small hair-like protrusions from the cell membrane known as microvilli[45] to more easily reach through the remaining ESG and bind to other sites on the HUVEC surface. For leukocytes in particular, L- and P-selectin are preferentially expressed on microvilli.[9,27,45] Therefore, if microvilli are able to infiltrate the reduced ESG layer, they may bind to selectin ligands that are expressed on the luminal surface of endothelial cells.[7] This however, does not fully explain why HA removal by hyaluronidase created the same adhesion enhancement as HS removal. Perhaps, under hyaluronidase treatment the ESG's structure is collapsed, thus exposing the same binding sites that cause the loss of anti-adhesive ability of the ESG under HS removal. Or, disruption of HA structure may expose new ligands for leukocyte adhesion molecules.

On activated HUVECs, however, the ESG itself participates in the adhesive interactions. When the HUVECs were stimulated with TNF-α, a significant increase in detachment force and work was observed. Upon treatment with heparinase I&III, the force was greatly reduced to similar levels observed in resting HUVECs that had been treated with heparinase I&III. This indicates that HS and HS proteoglycans may be the dominant ligands for leukocyte adhesion molecules that provide the vertical force component under TNF-α-stimulated conditions. Indeed HS has been reported by others to bind strongly to L-selectin,[19,31] and weakly to P-selectin.[19,30,31] Furthermore, TNF-α may provide import signals that trigger the modification of HS and HA. These factors play crucial roles in the initial selectin-dependent tethering step of leukocyte adhesion to the endothelial wall.

The processes of leukocyte adhesion make up a complex fourfold progression. The first step involves tethering due to L-, P- and E-selectins, which causes an initial deceleration under flow conditions.[7,12,37] In this study, we analyzed, *via* AFM, the separation forces that exist in cell–cell interactions between leukocytes and the endothelial cells on a short time scale of 0.5 s. This was to favor the first stage of leukocyte adhesion (Fig. 1) and avoid the second stage that involves further chemokines activation of leukocyte integrins,[37] which triggers firm adhesion *via* binding to ICAM-1 and VCAM-1.[4,8,34,37,52])

It is known that L-selectin binding to aortic endothelium can be strongly inhibited by heparinase I,

TABLE 1. TNF-α mediated ligand ruptures.

	TNF-α only	TNF-α hyaluronidase	TNF-α heparanases	TNF-α Hep/Hya
Average ruptures/scan	9.45 ± 0.276	6.91 ± 0.569	4.23 ± 0.817	4.23 ± 0.722
Average ruptures (μm^2)	0.544 ± 0.016	0.398 ± 0.034	0.244 ± 0.047	2.44 ± 0.042

	TNF-α (Anti-MYC on K562)	TNF-α (DREG.200 on K562)	TNF-α EDTA
Average ruptures/scan	9.57 ± 0.457	<2	<2
Average ruptures (μm^2)	0.551 ± 0.026	<0.05	<0.05

TABLE 2. Non-TNF-α mediated ligand ruptures.

	Untreated	Hyaluronidase	Heparanases
Average ruptures/scan	< 2	4.77 ± 0.436	5.88 ± 0.247
Average ruptures (μm^2)	<0.05	0.275 ± 0.025	0.339 ± 0.014
	Hep/Hya	EDTA	
Average ruptures/scan	5.29 ± 0.304	<2	
Average Ruptures (μm^2)	0.31 ± 0.012	<0.05	

II or III (inhibited by 50%) and by trypsin (inhibited by 90%), which is known to remove all of the cell surface constituents. This indicates that L-selectin binds to HS chains that are attached to HS proteoglycans[12] and other sites that are modulated by the contents of the cell surface. Consistently, we show that treatment of the K562s with DREG.200 almost completely inhibited their binding to HUVECs (Fig. 5). These findings lend support to previous experimental results from rolling assays[7,12] and further provide a disassociation force as well as the number of participating ligands in leukocyte-endothelial interactions.

Leukocyte-endothelial interaction is mediated by specific receptor-ligand interactions. Since the AFM is capable of detecting the unbinding force of single-molecular interactions, we sought to examine the number of rupture events per force scan during the separation process of each K562-HUVEC pair. The ligand rupture events were quantified for all experiments (Charts 1 and 2). Additionally, the number of ligand ruptures between the K562s and the HUVECs per μm^2 of contact were quantified as a function of the GAGs present on the cell surface (Fig. 7). The amount of GAGs that remained on cells was quantified from the WGA FITC fluorescence. Figure 7 clearly demonstrates the dual regulation of ESG in leukocyte adhesion and shows a clear decrease in ligand ruptures for the TNF-α-stimulated HUVECs as they are treated with hyaluronidase and/or heparanase I&III. Moreover, our data suggests that there are more active ligands that bind to L-selectin when endothelial cells are exposed to TNF-α and that this may be the cause for the increase in adhesive force seen experimentally (Tables 1, 2).

In summary, the data gathered from this study demonstrated the normal forces involved in the initial steps of leukocyte adhesion to the endothelial wall. The experiments were performed under cytokine-induced inflammatory response conditions and/or exposure to ESG digesting enzymes, such as heparinase I&III and/or hyaluronidase. We determined that the ESG layer serves two main functions. First, on resting endothelium, it prevents leukocyte adhesion, perhaps due to the electrostatic repulsion that the negatively charged GAGs have on the negatively charged leukocytes. Second, under inflammatory conditions, the ESG aggressively partakes in endothelial-leukocyte interactions, possibly *via* interactions between chemical moieties on GAGs and leukocyte adhesion molecules, such as selectins. These findings are among the first to investigate the forces involved in ESG-dependent leukocyte adhesion over short contact times *via* AFM. Knowledge of these forces will lead to a better understanding of pathological ESG shedding conditions and inflammatory responses in the human body.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (doi: 10.1007/s12195-016-0463-6) contains supplementary material, which is available to authorized users.

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CONFLICT OF INTEREST

Authors Matthew Dragovich, Kaylynn Genemaras, Hannah L. Dailey and Sabrina Jedlicka have no conflicts of interest to disclose. Author X. Frank Zhang has received funding from the American Heart Association (11SDG5420008) and Lehigh University (start-up funding) to conduct the research.

ETHICAL STATEMENT

Professors Zhang, Dailey, Jedlicka and Matthew Dragovich have no personal, professional or financial conflicts of interest in this project and/or proposal. We have conducted no research on human subjects, live or dead vertebrate animal subjects and our laboratories have accommodations for safe practice: Gloves, hoods, autoclaves, sterile equipment, proper refrigeration, etc..... All data acquisition for the applied force and work was gathered and analyzed in Igor; the figures seen in this paper of the AFM force-extension curves are renditions of the raw data gathered from Igor and are displayed in Origin. The Box and whiskers plots of the analyzed data are displayed in Excel. The equipment is owned by Professor Zhang and was purchased with his funding from Lehigh University. The equipment used in this project is managed by Mr. Dragovich with further oversight from Professor Zhang. Mr. Dragovich was responsible for gathering and analyzing the cell–cell adhesion data. The equipment in this project was also under use by the then undergraduate researcher Kaylynn Genemaras who was managed by Mr. Dragovich and assisted Mr. Dragovich in gathering and analyzing the cell–cell adhesion data. The AFM was previously used in collaboration with several other groups as well. Professor Dailey provided the statistical analysis for this project which is displayed in the figures and the appendix. Professor Jedlicka aided in providing the antibody staining images. The assertion that any publications from this project make will be handled appropriately so author integrity will be maintained. The data is presented with the expectation that it will be made public to accept challenges and/or corroborations. Since these findings may influence health care and be used by other scientific researchers they will particularly be handled with the upmost ethical responsibility. Intellectual property gained during this study from all participating in this project will be handled through the appropriate channels to be sure that appropriate credits and rights are obtained.

INFORMED CONSENT

No human studies were carried out by the authors for this article.

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