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Endothelial Cell Glycocalyx Modulates Immobilization of Leukocytes at the Endothelial Surface

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Objective—A thick endothelial glycocalyx provides the endothelial surface with a nonadherent shield. Oxidized LDL (Ox-LDL) degrades the endothelial glycocalyx. We hypothesized that glycocalyx degradation stimulates leukocyte–endothelial cell adhesion, whereas intravascular supplementation with sulfated polysaccharides reconstitutes the endothelial glycocalyx and attenuates Ox-LDL–induced leukocyte–endothelial cell adhesion.

Methods and Results—Degradation of the endothelial glycocalyx by local microinjection of heparitinase (10 to 50 U/mL) into mouse cremaster venules dose-dependently increased the number of adherent leukocytes. Systemic administration of Ox-LDL (0.4 mg/100 g body weight) induced 10.1±0.9 adherent leukocytes/100 μm at 60 minutes. In the venules perfused with 500-kDa dextran sulfate (1 mg/mL), the number of adherent leukocytes at 60 minutes after Ox-LDL bolus application was not influenced (9.2±1.0 leukocytes/100 μm). However, the venules locally perfused with heparan sulfate (10 mg/ml) or heparin (1 mg/ml) displayed a significantly lower number of adherent leukocytes induced by Ox-LDL: 5.1±0.7 and 5.4±0.9 leukocytes/100 μm, respectively (P<0.05). Fluorescently labeled heparan sulfate and heparin, but not dextran sulfate, attached to the venule luminal surface after Ox-LDL administration.

Conclusions—Endothelial glycocalyx degradation stimulates leukocyte immobilization at the endothelial surface. Circulating heparan sulfate and heparin attach to the venule wall and attenuate Ox-LDL–induced leukocyte immobilization. (Arterioscler Thromb Vasc Biol. 2003;23:1541-1547.)

Key Words: glycocalyx ■ leukocytes ■ oxidized LDL ■ heparin

The endothelial glycocalyx provides the endothelial surface with a negatively charged coating that contributes to the antiahesive nature of the endothelial cell surface.1,2 In the presence of inflammatory stimuli, the endothelial surface loses its nonadhesiveness because of the activation of adhesion molecules and becomes accessible to leukocytes. There is evidence that activation of adhesion molecules is associated with changes in the cell-surface glycocalyx.2–4 Indeed, the glycocalyx occupies a large domain at the endothelial surface,5,6 which most probably influences the adhesion process. In this investigation, we studied the effect of endothelial glycocalyx condition on leukocyte–endothelial cell adhesion.

Understanding the role of the endothelial glycocalyx in leukocyte–endothelial cell adhesion has direct relevance for atherosclerosis-related conditions, such as hypercholesterolemia and the plasma presence of oxidized lipoproteins, which are associated with increased leukocyte recruitment7,8 and degradation of the endothelial glycocalyx.9–11 Noteworthy, early modifications of endothelial cells during diet-induced hypercholesterolemia are represented by a decreased thickness and anionic charge of the endothelial glycocalyx and by changes in its biochemical composition.9,10 Degradation of the endothelial glycocalyx is also induced by oxidized LDL (Ox-LDL in clinically relevant doses and is associated with a parallel increase in endothelial surface adhesiveness.11

The first aim of the present study was to induce a primary alteration of the thickness and charge of the endothelial glycocalyx by enzyme treatment and to investigate its effect on leukocyte rolling and adhesion. Because heparan sulfate proteoglycans represent an abundant, highly negatively charged constituent of the endothelial glycocalyx,12,13 we administered heparitinase (heparinase III) by intraluminal microperfusion into mouse cremaster muscle venules.

In the second part of the study, we hypothesized that maintenance of the endothelial glycocalyx during an Ox-LDL challenge prevents Ox-LDL–induced adhesiveness. To test this hypothesis, we supplemented exogenous heparan sulfate with intraluminal microperfusion in mouse cremaster venules before systemic bolus administration of Ox-LDL. However, because sulfated polysaccharides, including heparan sulfate, are able to inhibit selectin-mediated leukocyte rolling by acting directly on leukocytes,14 we had to differentiate between the inhibition of leukocyte rolling and the reconstitution of the endothelial glycocalyx after heparan sulfate administration. Therefore, we additionally determined the effect of other sulfated polysaccharides that are not constituents of the glycocalyx, ie, heparin and 500-kDa dextran.
sulfate, on Ox-LDL–induced leukocyte rolling and adhesion. For all 3 sulfated polysaccharides, binding to the endothelial surface and thereby, glyocalyx reconstitution were studied by using fluorescently labeled forms of their molecules.

Methods

Animal Preparation and Intravital Microscopy

Male C57BL/6 mice with a body weight (BW) range of 20 to 30 g (n = 39) were anesthetized with a single intraperitoneal injection of ketamine hydrochloride (125 mg/kg BW) and xylazine (7.5 mg/kg BW). Anesthesia was maintained with intraperitoneal injections of ketamine hydrochloride (15 mg/kg BW) administered at 1-hour intervals. The trachea was cannulated to ensure a patent airway, and the jugular vein was cannulated for injection of Ox-LDL. Body temperature was maintained at 37°C with a heating lamp. The right cremaster muscle was prepared by longitudinal incision without cutting the connection to the epididymis. The muscle was continuously superfused at 34°C (5 mL/min) with a bicarbonate-buffered physiologic salt solution previously described. All procedures were approved by the local animal ethics committee. Venules (20 to 60 μm) of the cremaster muscle were examined with an intravital microscope (Olympus BHM) with a ×20 objective lens (Olympus MSPlan 20, numerical aperture, 0.40) by transillumination with a mercury lamp (100 W).

Ox-LDL Preparation

Human LDL (Sigma, L2139) was dialyzed against phosphate-buffered saline (PBS) at pH 7.4 at 4°C for 24 hours. Oxidation of LDL was achieved by addition of CuSO4 to a final concentration of 10 μmol/L and incubation at 37°C for 24 hours. The oxidative reaction was stopped by addition of EDTA to a final concentration of 0.01 mmol/L, and the Ox-LDL sample was further dialyzed in PBS plus 0.01% EDTA at 4°C for 48 hours. The efficacy of LDL oxidation was determined by analyzing the content of thiobarbituric acid–reactive substances of the sample, expressed as malondialdehyde equivalents, which averaged 7.6 ± 0.9 nmol malondialdehyde equivalents per milligram protein (compared with 0.03 ± 0.01 nmol malondialdehyde equivalents/mg protein in nonoxidized LDL). Ox-LDL protein concentration averaged 5.3 ± 0.3 mg/mL.

Microapplications

Micropipettes drawn from borosilicate glass capillary tubes (outer diameter, 1 mm) were beveled to a sharp tip of 7 to 8 μm. Micropipettes were sterilized by autoclaving for at least 1 hour and rinsed with distilled water. The right cremaster venule was cannulated for injection of Ox-LDL. Body temperature was maintained at 37°C with a heating lamp. The right cremaster muscle was prepared by longitudinal incision without cutting the connection to the epididymis. The muscle was continuously superfused at 34°C (5 mL/min) with a bicarbonate-buffered physiologic salt solution previously described. All procedures were approved by the local animal ethics committee. Venules (20 to 60 μm) of the cremaster muscle were examined with an intravital microscope (Olympus BHM) with a ×20 objective lens (Olympus MSPlan 20, numerical aperture, 0.40) by transillumination with a mercury lamp (100 W).

Experimental Protocol

Baseline values for rolling and adherent leukocytes were obtained before intraluminal microapplications. The microperfused venule, 1 per animal, was examined and recorded continuously from the beginning of microperfusion at a distance of >1000 μm from the site of micropipette insertion to avoid interference with insertion-induced local inflammation. One of the following treatments was applied in each experiment: (1) Microperfusion of heparitinase for 30 minutes at 1 of the following concentrations: 0 U/mL (vehicle, n = 4), 10 U/mL (n = 3), 20 U/mL (n = 3), and 50 U/mL (n = 4). (2) Microperfusion of vehicle (n = 4), heparan sulfate 10 mg/mL (n = 4), heparin 1 mg/mL (n = 4), and dextran sulfate 1 mg/mL (n = 4) for 30 minutes before systemic bolus of 0.4 mg/100 g BW Ox-LDL. Microperfusion was continued for another 30 minutes in the presence of Ox-LDL. In each animal, at least 1 venule other than the microperfused venule was recorded under control conditions and after 60 minutes from the moment of heparitinase microapplication or Ox-LDL bolus injection and served as a paired control for each experiment.

Blood samples were collected from mouse tail veins under baseline conditions and after 30 minutes of systemic Ox-LDL injection in 10 experiments and were analyzed with a cell counter (Coulter) to obtain systemic leukocyte counts. Erythrocyte centerline velocity was measured online with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University) connected to acquisition software (Chart for Windows). Images were recorded on an SVHS videotape recorder (JVC BR-S611E) with a charge-coupled-device video camera and a time-coding interface unit (JVC SA-F911E) for data analysis.

Fluorescence Microscopy

The binding of sulfated polysaccharides to the endothelial surface was assessed in additional experiments by intravital fluorescence microscopy. Fluorescein-conjugated heparan sulfate (Sigma H7640) was custom-made by Molecular Probes (1.5 mol dye/mol; concentration, 1.25 mg/mL PBS) and was diluted with vehicle solution containing 5% albumin to a concentration 1 mg/mL in the micropipette. Microperfuions with fluorescein-conjugated heparan sulfate (n = 3), fluorescein-conjugated heparin (n = 3), and fluorescein-conjugated dextran sulfate (n = 3) were performed for 30 minutes before and were continued for 30 minutes after systemic Ox-LDL administration. Fluorescence recordings were made with a fast, high-resolution, low-light-performance digital camera with on-chip multiplex gain (Photometrics Cascade, Roper Scientific BV) and were analyzed with data acquisition software (METAMORPH version 5.0r4, Universal Imaging Corp).

Data Analysis

Video images were analyzed as reported. Wall shear rate in the examined venule was calculated by using the formula 2.12(8V/D), where 2.12 is a median empirical correction factor obtained from actual velocity profiles measured in microvessels in vivo. V represents the mean blood flow velocity, and D is the venular diameter. Mean blood flow velocity was calculating by dividing erythrocyte centerline velocity by an empirical factor of 1.6.

Rolling leukocyte flux was determined offline as the number of rolling leukocytes passing a specified point in the venule for each minute. Rolling velocity was normalized to rolling leukocyte to traverse 100 μm of the venule, and 5 to 7 measurements were averaged per minute. The measurements were averaged over intervals of 5 minutes and normalized to baseline values. Leukocytes that remained stationary for >30 seconds were measured every minute and averaged over a 5-minute period as adherent leukocytes 100 μm of venule length.

Data are presented as mean±SEM for the indicated number of experiments. Data between groups were compared with a univariate general linear model, followed by a Bonferroni post hoc test (SPSS 10.1 software) after the following case selection: the period of microperfusion (before/after Ox-LDL) and the period after microperfusion cessation. A value of P < 0.05 was considered statistically significant.
Results
Systemic leukocyte count decreased after Ox-LDL administration, from 5730±420 to 4840±403 cells/μL (paired t test, \( P<0.05 \)). No significant differences regarding baseline diameter, shear rate, leukocyte rolling flux, and velocity in the microperfused venules were found between groups (please see Table I in the online data supplement at http://www.ahajournals.org). In brief, baseline venule diameter and shear rate corresponding to each group were as follows: 35.4±5.0 μm and 1070±115 s\(^{-1}\) in the vehicle (0 U/mL heparitinase) group (n=4); 30.6±3.5 μm and 1285±130 s\(^{-1}\) in the 10 U/mL heparitinase group (n=3); 36.0±3.8 μm and 1221±145 s\(^{-1}\) in the 20 U/mL heparitinase group (n=3); 33.9±3.0 μm and 1118±73 s\(^{-1}\) in the 50 U/mL heparitinase group (n=4); 45.5±4.2 μm and 1151±65 s\(^{-1}\) in the Ox-LDL+vehicle group (n=4); 37.5±4.4 μm and 1150±175 s\(^{-1}\) in the Ox-LDL+10 mg/mL heparan sulfate group (n=4); 44.3±7.2 μm and 1162±152 s\(^{-1}\) in the Ox-LDL+1 mg/mL heparin group (n=4); and 34.1±5.3 μm and 1274±160 s\(^{-1}\) in the Ox-LDL+1 mg/mL dextran sulfate group (n=4).

Heparitinase Microperfusion
At heparitinase concentrations of 10 and 20 U/mL, leukocyte rolling velocity (Figure 1, top) and rolling flux (Figure 1, bottom) were not affected. At a concentration of 50 U/mL heparitinase, leukocyte rolling velocity and flux decreased compared with control vehicle microperfusion.

At all 3 heparitinase concentrations, the number of adherent leukocytes was significantly increased compared with vehicle microperfusion. This increase was dose dependent (Figure 2, top). At 60 minutes, there were 2.8±0.4 adherent leukocytes/100 μm after vehicle microperfusion. In comparison, heparitinase at the concentrations of 10, 20, and 50 U/mL induced 5.5±0.4, 9.1±1.3, and 16.9±1.3 adherent leukocytes/100 μm, respectively (\( P<0.05 \) for each concentration compared with vehicle). In the venules receiving no treatment (microperfusion) that served as paired controls in each experiment, there were 3.6±0.2 adherent leukocytes/100 μm at 60 minutes (averaged over all 14 heparitinase experiments), significantly less than in the venules perfused with heparitinase at all 3 concentrations. The shear rate in the microperfused venules did not present significant variation between groups or when compared with baseline (please see Figure I in online data supplement at http://www.ahajournals.org).

Microperfusion of Sulfated Polysaccharides and Systemic Administration of Ox-LDL

Leukocyte Rolling Flux and Velocity
Thirty minutes after the start of microperfusion with sulfated polysaccharides, Ox-LDL was given and microperfusion continued for 30 minutes. Leukocyte rolling velocity was higher (dextran sulfate) or similar (heparan sulfate and heparin) to that of vehicle-perfused venules before Ox-LDL administration (Figure 3, top). However, leukocyte rolling flux decreased significantly during microperfusion with all 3 sulfated polysaccharides: 53.4±6.5% for heparan sulfate, 55.4±7.1% for heparin, and 27.2±6.2% for dextran sulfate at 30 minutes (Figure 3, bottom).

Systemic administration of Ox-LDL significantly decreased leukocyte rolling velocity to 50.9±8.7% (at 30 minutes; \( P<0.05 \)) in the venules microperfused with vehicle. Rolling flux decreased gradually to 45.5±6.4% at 30 minutes. In contrast, Ox-LDL did not decrease leukocyte rolling velocity during microperfusion with the sulfated polysaccharides, which remained significantly higher compared with the vehicle- and Ox-LDL–perfused venules.

Although leukocyte rolling velocity was maintained by the microperfusion with sulfated polysaccharides during the first 30 minutes of Ox-LDL challenge, it dropped markedly after microperfusion cessation to values that were no different from those for vehicle plus Ox-LDL. Leukocyte rolling flux after cessation of microperfusion remained significantly higher in the venules exposed to heparan sulfate and heparin compared with vehicle.

Leukocyte Firm Adhesion
After 30 minutes of Ox-LDL challenge, the number of adhering leukocytes increased in the case of vehicle perfusion.
to a higher level than with microperfusion with the 3 sulfated polysaccharides (Figure 4, top). After cessation of microperfusion, at 60 minutes after Ox-LDL bolus, the number of adherent leukocytes increased to 10.1 ± 0.9 leukocytes/100 μm in the vehicle-perfused venules. In the venules perfused with heparan sulfate and heparin, Ox-LDL induced 5.1 ± 0.7 and 5.4 ± 0.9 adherent leukocytes/100 μm, respectively, at 60 minutes, significantly less compared with vehicle-perfused venules (P < 0.05). In contrast, the number of adherent leukocytes at 60 minutes after Ox-LDL challenge in the venules perfused with dextran sulfate was 9.2 ± 1.0 leukocytes/100 μm, a value not significantly different from that for vehicle-perfused venules (P > 0.05).

The shear rate in the microperfused venules did not significantly vary between groups or when compared with baseline (please see Figure II in the online data supplement at http://www.ahajournals.org).

**Binding of Sulfated Polysaccharides at the Venule Luminal Surface**

In additional experiments, an increase in fluorescence at the venule wall was observed after systemic administration of Ox-LDL in the venules perfused with dextran sulfate (n=3) and heparin (n=3; figure 5) but not in the venules perfused with fluorescein-conjugated dextran sulfate (n=3). The increases in fluorescence were observed between consecutive microinjection pulses, after washout of the dye from the venule lumen by flowing blood. After microperfusion ended, the increase in fluorescence at the vessel wall persisted for the remainder of the observation period (60 minutes after Ox-LDL bolus).

**Discussion**

Leukocyte adhesion to endothelial cells is a complex process that involves capture of free-flowing leukocytes from the bloodstream, rolling on the endothelial surface, deceleration, and eventually, leukocyte immobilization (firm adhesion). We found that degradation of the endothelial glycocalyx by heparitinase at low concentrations stimulated immobilization of leukocytes in the absence of changes in leukocyte rolling behavior.

Heparan sulfate, heparin, and dextran sulfate prevented the Ox-LDL–induced deceleration of rolling leukocytes and leu-
Leukocyte Adhesion After Endothelial Glycocalyx Degradation by Heparitinase

The endothelial glycocalyx consists of membrane-attached proteoglycans and glycoproteins, which form a negatively charged matrix covering the endothelial cell surface. Heparan sulfate proteoglycans are abundant on the endothelial cell surface, and bind various plasma proteins, therefore contributing to a major part of the glycocalyx charge and thickness. The total thickness of the glycocalyx extends >0.5 μm, as observed in vivo by measuring the distance of erythrocytes to the capillary endothelial surface and as recently confirmed by electron microscopy after application of Alcian blue stain.

Heparitinase treatment was used to reduce glycocalyx thickness and negative charge. At low concentrations of heparitinase (10 to 20 U/mL), the number of stationary leukocytes increased in the absence of significant changes in leukocyte rolling velocity or flux. At 50 U/mL heparitinase, a significant decrease in rolling velocity and flux occurred, probably due to leukocyte-leukocyte interaction with the large number of stationary leukocytes.

The absence of an effect on leukocyte rolling at low concentrations of heparitinase might be explained by localization of rolling molecules on the tips of leukocyte microvilli. These microvilli can penetrate the endothelial glycocalyx matrix and thereby inducing rearrangement of glycocalyx matrix, heparitinase is likely to increase the access of heparan sulfate proteoglycans and glycoproteins and adsorbed plasma proteins, which form a negatively charged matrix covering the endothelial cell surface.

Figure 4. Number of adherent leukocytes was significantly lower in venules perfused with dextran sulfate compared with vehicle (α) before Ox-LDL administration. After Ox-LDL administration, number of adherent leukocytes remained significantly lower during perfusion with heparan sulfate (*), heparin (#), and dextran sulfate compared with vehicle. After microperfusion cessation, number of adherent leukocytes remained significantly lower only in venules perfused with heparan sulfate and heparin.

kocyte immobilization during the period of microperfusion. After cessation of microperfusion, immobilization of leukocytes at the endothelial surface remained decreased in the venules perfused with heparan sulfate and heparin but not in those perfused with dextran sulfate. This paralleled the finding that heparan sulfate and heparin remained attached to the vessel wall.

Figure 5. A–C, Heparan sulfate–perfused venule (60.2 μm) observed during microperfusion by transillumination (A) and fluorescence microscopy (B). Note that fluorescein-conjugated heparan sulfate did not entirely fill the lumen. C, Persistence of fluorescence at the perfused side of the vessel wall after microperfusion cessation at 30 minutes from Ox-LDL bolus administration. D–F, Heparin–perfused venule (56.5 μm) observed by transillumination (D) and fluorescence microscopy (E) during microperfusion that remained confined to the lower half of venule lumen. F, Comparison between increased fluorescence intensity at the perfused luminal border compared with the nonperfused border that displays only autofluorescence, after microperfusion cessation at 30 minutes from Ox-LDL bolus.
adhesion cascade. In contrast, in the present study, selective removal of heparan sulfate proteoglycans primarily increased leukocyte immobilization in the absence of changes in leukocyte rolling.

**Contribution of Glycocalyx Degradation to the Proadhesive Effect of Ox-LDL**

Consistent with other reports,8,20 Ox-LDL considerably increased the number of stationary leukocytes at the endothelial surface. It has been shown that Ox-LDL is associated with increased expression of several rolling and adhesion molecules: P-selectins, L-selectins, ICAM-1, and CD11/CD18.20 Thus, the decrease in leukocyte rolling flux after Ox-LDL administration in the present study might seem paradoxical, but it paralleled the decrease in rolling velocity that preceded immobilization. Furthermore, systemic leukocyte count decreased after Ox-LDL bolus, indicating that leukocyte immobilization occurred in multiple organs, which probably induced depletion of the leukocyte rolling stream.

Although Ox-LDL increases expression of endothelial adhesion molecules, degradation of the endothelial glycocalyx might represent a common pathway by which Ox-LDL and heparitinase stimulate immobilization of leukocytes at the endothelial surface. This notion is consistent with biochemical studies showing that exposure of endothelial cells to Ox-LDL decreases the amount of heparan sulfate proteoglycans associated with the cell surface.21,22 Therefore, we hypothesized that supplementation with exogenous heparan sulfate might increase the thickness and negative charge of the endothelial glycocalyx and thereby prevent leukocyte immobilization.

It is known that sulfated polysaccharides inhibit leukocyte rolling by competing with the binding of L-selectins to endothelial ligands.23 Ley et al14 reported that their inhibitory potency is different, as follows: 500-kDa dextran sulfate>heparin>heparan sulfate. Therefore, we used higher doses of heparan sulfate (10 mg/mL) than dextran sulfate and heparin (1 mg/mL) to investigate leukocyte rolling and stationary adhesion. However, binding of heparan sulfate to the endothelial surface was already observed at a concentration of 1 mg/mL, at which the custom-made fluorescent form of heparan sulfate was delivered.

To differentiate between inhibition of leukocyte stationary adhesion caused by rolling inhibition versus that by glycocalyx reconstitution, the micropulsed venules were also observed after cessation of microperfusion. Leukocyte rolling behavior was not inhibited longer after cessation of micropulsed perfusion with all 3 sulfated polysaccharides and was similar to that in vehicle- plus Ox-LDL–perfused venules. Despite this, inhibition of leukocyte immobilization continued in the venules perfused with heparan sulfate and heparin. This was most likely due to reconstitution of the glycocalyx matrix induced by the attachment of heparan sulfate and heparin to the vessel wall.

Although heparin is not a natural component of the endothelial cell glycocalyx, its structure shows a high resemblance to heparan sulfate glycosaminoglycans that are produced by endothelial cells.13 Therefore, circulating heparin might replace heparan sulfate glycosaminoglycans at vascular sites where the glycocalyx is degraded. Binding of heparin to the endothelial surface in cell cultures or after injection into the circulation has also been reported in earlier studies.24,25 Administration of 500-kDa dextran sulfate served eventually as a negative control for the effect of glycocalyx reconstitution on leukocyte adhesion, because it inhibited leukocyte immobilization as a consequence of rolling inhibition, but only during micropulsation.

**Conclusion**

The present study shows that disruption of heparan sulfate proteoglycans of the endothelial glycocalyx stimulates firm adhesion of leukocytes. Intravascular administration of heparan sulfate and heparin not only decreased leukocyte rolling by competing with rolling molecules but also prevented leukocyte immobilization by binding to the endothelial surface and thereby increasing the thickness and negative charge of the endothelial glycocalyx. The mechanisms responsible for the binding of heparan sulfate and heparin at the endothelial surface are not known but might involve interactions with membrane-bound glycosaminoglycans and proteins, as in the case of extracellular matrix formation.13 These mechanisms appear to be stimulated by increased oxidative stress, as induced by the plasma presence of Ox-LDL in the present study.

**References**


