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Oxidized Lipoproteins Degrade the Endothelial Surface Layer Implications for Platelet-Endothelial Cell Adhesion

Hans Vink, PhD; Alina A. Constantinescu, MD; Jos A.E. Spaan, PhD

Background—Flowing erythrocytes and platelets are separated from the luminal endothelial cell (EC) surface by a 0.5- μm -wide space named the endothelial surface layer. We hypothesized that the disruption of the endothelial surface layer by oxidized low-density lipoproteins (Ox-LDL) contributes to atherogenic increases in vascular wall adhesiveness.

Methods and Results—The hamster cremaster muscle preparation was used for intravital microscopic observation of the distance between erythrocytes and the capillary EC surface. Moderate Ox-LDL was prepared by exposing native LDL to CuSO_4 for 6 hours. The dimension of the EC surface layer averaged $0.6 \pm 0.1 \mu\text{m}$ during control situations, but a bolus intravenous injection of Ox-LDL (0.4 mg/100 g of body weight) transiently diminished the EC surface layer by 60% within 25 minutes, which correlated with a transient increase in the number of platelet-EC adhesions. Combined administration of superoxide dismutase and catalase completely blocked the effect of Ox-LDL on the dimension of the EC surface layer and inhibited platelet-EC adhesion.

Conclusions—Oxygen-derived free radicals mediate the disruption of the EC surface layer and increase vascular wall adhesiveness by Ox-LDL. (*Circulation*. 2000;101:1500-1502.)

Key Words: atherosclerosis ■ lipoproteins ■ endothelium ■ platelets

Damage to the endothelial cell (EC) glycocalyx seems to be the earliest detectable injury to the vascular wall during the development of atherosclerosis and is associated with increased vascular permeability and adhesiveness.^{1,2} Recently, Vink and Duling³ showed that measuring the relative positions of flowing blood cells and the EC membrane in skeletal muscle capillaries provided dimensional information on the in vivo endothelial surface layer, which includes the EC glycocalyx and associated plasma proteins. Moreover, they demonstrated that light-dye-induced generation of oxygen-derived free radicals disrupted the endothelial surface layer, which resulted in localized adhesion of platelets and red blood cells (RBCs) to the vascular wall.³ In the present study, we tested the hypothesis that oxidized low-density lipoproteins (Ox-LDL) modulate the endothelial surface layer in a similar fashion.

Methods

Animal Preparation

Male, Golden hamsters (n=19; body weight, 139 ± 7 g) were anesthetized with intraperitoneal pentobarbital sodium (70 mg/kg body weight), and the trachea was cannulated to ensure a patent airway. The left femoral vein was cannulated for the continuous infusion of 0.9% saline (0.5 mL/h) containing 10 mg/mL pentobarbital sodium. The hamster was placed on a plexiglas platter, and the right cremaster muscle was prepared for visualization of the micro-

circulation as previously described.³ The cremaster muscle was continuously superfused at 5 mL/min with a bicarbonate-buffered physiological salt solution (composition [in mmol/L]: NaCl 131.9, KCl 4.6, CaCl_2 2.0, MgSO_4 1.2, and NaHCO_3 20). The superfusion solution was gas-equilibrated with 5% CO_2 and 95% N_2 to obtain a pH of 7.35 to 7.45, and the solution was maintained at 34°C. Succinylcholine (10^{-5} mol/L; Sigma) was added to the superfusion solution to reduce spontaneous skeletal muscle contractions. Body temperature was maintained at 37°C to 38°C with conducted heat. These procedures were performed in accordance with institutional guidelines.

Intravital Microscopy

The cremaster muscle was observed with an intravital microscope (Olympus BHM) and a cooled intensified charge-coupled device video camera (GenIV ICCD, Princeton Instruments). The tissue was transilluminated with a mercury lamp (100 W) equipped with a 435-nm bandpass interference filter (blue light) using an applanate, achromatic condenser set at numerical aperture 1.2 (Universal Applanate Achromatic Condenser, Olympus). All preparations were examined with a $\times 60$, water-immersion, objective lens (Olympus, UPlanApo with aperture of 1.2 W or LUMPlanFL with aperture of 0.9 W) and a telescopic tube to give a final object-to-camera magnification of $\times 250$. Images were displayed on a Philips CM 8833-II video monitor and recorded using a SVHS videotape recorder (JVC BR-S611E) and a time-coding interface unit (JVC SA-F911E) for further image analysis.

Data Analysis

Video images were digitized using a frame grabber (DT3152, PCI Local Bus) and Image-Pro Plus software (Image-Pro Plus version

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3.0, Media Cybernetics). An onscreen caliper using a 1 mm/0.01 mm stage micrometer was used for all calibrated dimensional measurements. The anatomical capillary diameter was estimated by positioning the digital calipers at the inside of the capillary wall. Observed microvessel diameters ranged between 3 and 10 μm , indicating that in addition to true capillary blood vessels, the observed population of microvessels probably included terminal arterioles and/or capillary venules. Platelets that remained stuck to the endothelium for ≥ 2 video frames in the presence of continuously flowing RBCs were counted off-line by slow motion video play back.

Experimental Protocols

All experimental protocols started 45 to 60 minutes after completion of the hamster cremaster preparation. Measurements of capillary dimensions were made starting 10 to 15 minutes before the injection of either native LDL (n=4) or Ox-LDL (n=7) at 0.4 mg/100 g body weight. Human LDL (Sigma, L2139) was dialyzed against PBS for 24 hours at 4°C at pH 7.4 without EDTA. LDL was oxidized by the addition of CuSO_4 at a concentration of 7.5 $\mu\text{mol/L}$ for 6 hours at 37°C. This reaction was stopped by the addition of 0.01 mmol/L EDTA; normal LDL was stored for 6 hours at 4°C. Finally, both normal LDL and Ox-LDL were dialyzed for 48 hours in PBS and 0.01% EDTA at 4°C. Protein concentrations were determined using the method of Lowry et al.^{3a} LDL samples were stored at 4°C. Using a measurement of ≈ 5 mL of plasma volume in a 100-g hamster, the initial systemic Ox-LDL concentration was estimated as ≤ 0.4 mg/5 mL, or ≤ 8 mg/dL. This measurement seems to be clinically relevant on the basis of recent measurements in atherosclerotic patients; these measurements show Ox-LDL concentrations between 1 and 6 mg/dL.⁴

To test for the involvement of oxygen-derived free radicals, additional Ox-LDL experiments (n=8) were performed in the presence of superoxide dismutase (SOD) and catalase, as described previously.³

Statistics

Data on the dimension of the spacing between RBCs and the luminal EC membrane (RBC-EC gap) are presented as means \pm SE. RBC-EC gap values after the injection of either normal LDL or Ox-LDL were compared with their respective controls (preinjection values) using a paired *t* test (2-way) to test for significance at $P < 0.05$.

Results

Figure 1 shows the gap between flowing RBCs and the luminal EC membrane as a measure of the dimension of the endothelial surface layer. The injection of moderate Ox-LDL (Figure 1B), but not normal LDL (Figure 1A), resulted in a transient decrease of $>50\%$ in the distance of flowing blood cells to the endothelial surface, which could be completely blocked by the administration of SOD and catalase (Figure 1C). Numbers of adhering platelets counted in 10-minute intervals are depicted on the time axis. Only 1 platelet was seen adhering spontaneously to the EC surface in a total of 4 experiments after the injection of normal LDL. In contrast, in 7 of 7 Ox-LDL experiments, ≥ 1 platelet was seen sticking to the endothelium, giving a total of 15 platelet-EC adhesions in 7 experiments with Ox-LDL. No adhering platelets were observed in the presence of SOD and catalase. Figure 2 depicts examples of the transient decrease in RBC-EC gap dimensions at 0, 24, and 70 minutes after bolus injection of Ox-LDL.

Discussion

Although early electron microscopic studies revealed decades ago that carbohydrate-rich endothelial surface structures form the interface between blood and the luminal endothelial

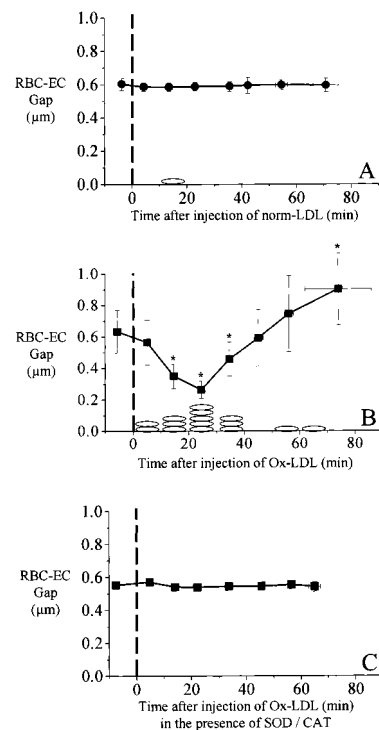


Figure 1. Dimension of gap between flowing RBCs and luminal EC membrane as a measure of dimension of endothelial surface layer, before and after injection of either normal LDL (A), Ox-LDL (B), or Ox-LDL together with SOD and catalase (C). Platelets observed to adhere to EC surface within 10-minute intervals are depicted on time axis as spheres. *Significantly different ($P < 0.05$) from value before injection of Ox-LDL.

membrane,⁵ relatively few studies have attempted to associate a modulation of EC function with a modification of the endothelial surface layer, which is defined as the EC glycocalyx and associated plasma proteins.

Treating the RBC surface charge with polycations⁶ or enzymatically treating the endothelial surface layer^{7,8} modulates RBC flow through capillary blood vessels and stimulates platelet-EC adhesion. Studies on transvascular exchange demonstrated that the adsorption of albumin and other plasma proteins to the EC glycocalyx confers filter-like properties to the endothelial surface layer and reduces microvascular hydraulic conductivity and solute permeability.^{9–11} Enzymatic disruption of the EC glycocalyx increased the adhesion of blood cells to the vascular wall⁸ and abolished flow-dependent dilation due to impaired endothelial production of nitric oxide.^{12,13} These studies demonstrate that the endothelial surface layer is essential for several aspects of endothelial function, including the control of transvascular exchange, providing vessels with an anti-adhesive inner lining, and flow-dependent dilation.

In the present study, we used a recently developed light microscopic technique³ to demonstrate for the first time that a clinically relevant dose of Ox-LDL⁴ reduces the in vivo dimension of the endothelial surface layer and simultaneously increases platelet-EC adhesion. These effects were completely inhibited by the administration of SOD and catalase, indicating that increased amounts of oxygen-derived free radicals mediate Ox-LDL-induced degradation of the endo-

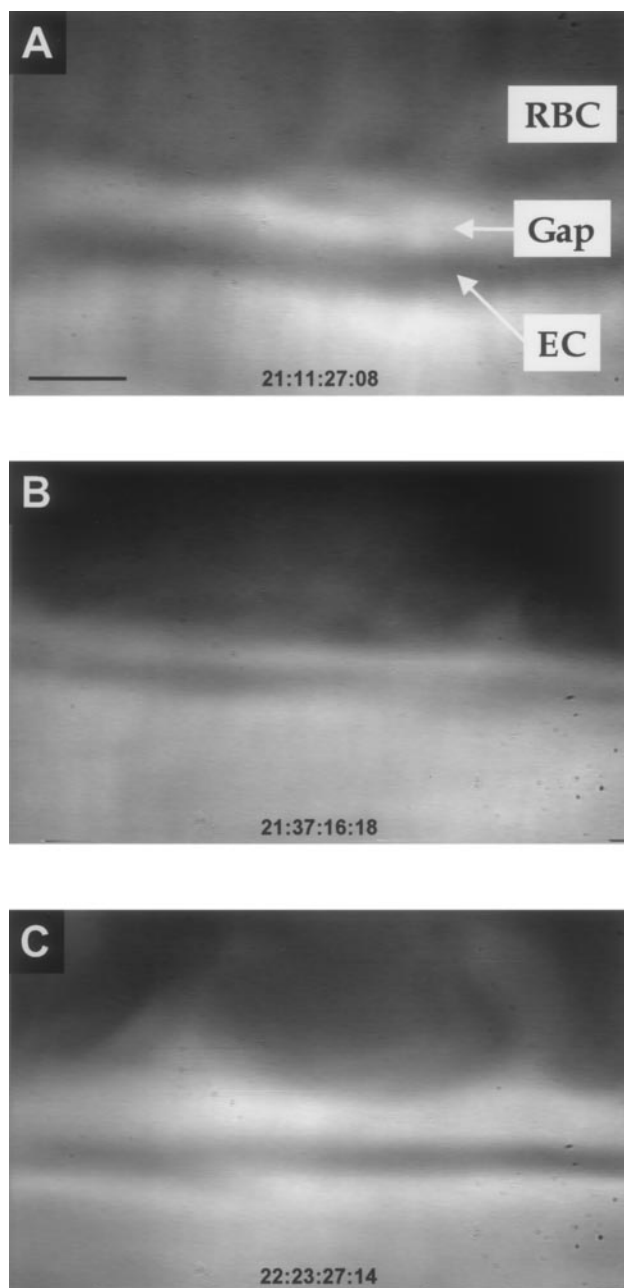


Figure 2. Examples of light microscopic images of RBCs flowing through a hamster cremaster muscle capillary during an Ox-LDL experiment, showing gap between flowing RBCs and luminal EC membrane. A, B, and C show transient decrease in RBC-EC gap dimension at 0, 24, and 70 minutes, respectively, after bolus injection of Ox-LDL. Calibration bar in A represents 2 μm .

thelial surface layer and the consequent loss of endothelial anti-adhesive properties. Similarly, Lehr et al¹⁴ demonstrated that the increased adhesion of leukocytes to the endothelium of small arterioles and venules after the systemic bolus injection of Ox-LDL could be prevented by vitamin C or SOD. Furthermore, Liao and Granger¹⁵ showed that SOD prevented Ox-LDL-induced albumin leakage and leukocyte adhesion to the vascular endothelium.

Oxygen-derived free radicals such as the superoxide anion may degrade the endothelial surface layer and induce the adhesion of platelets by inactivating paracrine anti-platelet sub-

stances, such as nitric oxide. Alternatively, oxygen radicals may have unmasked constitutive endothelial adhesion molecules, such as platelet-endothelial cell adhesion molecule, by removing glycocalyx-associated plasma proteins from the EC surface. Readsorption of plasma substances to the EC glycocalyx might then explain the rapid recovery of the endothelial surface layer after its disruption by a bolus injection of Ox-LDL. In agreement with this possibility, our reported layer recovery time (20 to 30 minutes) agrees well with published data on plasma protein reconstitution of the EC surface layer after vascular perfusion with a protein-poor medium.¹⁶ However, reconstitution of the EC surface layer by newly synthesized or preformed proteoglycans and/or glycosaminoglycans must also be considered, and additional studies must be designed to distinguish between these mechanisms of surface layer repair.

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