Oxidized LDL-Induced Leukocyte/Endothelium Interaction In Vivo Involves the Receptor for Platelet-Activating Factor

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Leukocyte adhesion and subendothelial emigration, constant hallmarks of early atherogenesis, have been ascribed to the action of oxidized low-density lipoprotein (oxLDL). Using intravital fluorescence microscopy in the skinfold-chamber model in hamsters, we have previously shown that systemic administration of oxLDL stimulates leukocyte adhesion in vivo through a mechanism that depends on the generation and/or action of both leukotrienes and superoxide radicals. On the basis of the fact that oxygen radical-catalyzed peroxidation of phospholipids results in the generation of fragments with short sn2 residues, which besides authentic platelet-activating factor (PAF), activate the receptor for PAF on leukocytes and thereby induce leukocyte adhesion, we asked whether pretreatment of hamsters with a specific PAF receptor antagonist (WEB2170; 1 mg/kg of body weight IV, 10 minutes before oxLDL) attenuates leukocyte adhesion after injection of oxLDL (4 mg/kg of body weight IV, oxidized by 7.5 μmol/L Cu2+ for 18 hours at 37°C). We demonstrate herein that in contrast to untreated control animals in which oxLDL elicited rolling and adhesion of circulating leukocytes to the endothelium of venules and arterioles, oxLDL-induced leukocyte adhesion was significantly attenuated in WEB2170-pretreated animals. These changes cannot be ascribed to alterations of microhemodynamic parameters and, hence, wall shear conditions. This finding indicates that oxLDL-induced leukocyte/endothelium interaction involves the PAF receptor, which may function both as a receptor for authentic PAF or for PAF-like lipids that are generated in a free radical-catalyzed peroxidation of phospholipids. (Arteriosclerosis and Thrombosis 1993;13:1013-1018)

KEY WORDS • LDL • platelet-activating factor • microcirculation • leukocyte/endothelium interaction • atherosclerosis

Growing evidence has accumulated within the last few years for an oxidative modification hypothesis of atherogenesis, ie, that oxygen radicals contribute to the sequelae of events leading to the inception and progression of atherosclerotic lesions (reviewed in Reference 1). One well-established consequence of oxygen radical–induced lipid peroxidation under various pathological conditions is the generation of oxidized low-density lipoprotein (oxLDL) within the vessel wall or even within the bloodstream.3,4

In vitro and in vivo experiments have demonstrated that oxLDL induces the chemotactic accumulation and adhesion of leukocytes to endothelial cells.5-8 Both the peroxidative modification of LDL9,10 and the stimulation of leukocyte adhesion by oxLDL11 can be inhibited by superoxide dismutase (SOD), thus suggesting that these events involve a superoxide radical–dependent peroxidation of LDL and membrane phospholipids.

Uncontrolled oxygen radical–catalyzed peroxidation of phospholipids results in the generation of numerous fragmented phospholipids, some of which activate leukocytes.12 More recent in vitro experiments have demonstrated that oxidatively fragmented phospholipids with a short sn2 polyunsaturated fatty acid residue, derived from either synthetic phosphatidylcholine12 or membrane phospholipids of viable cells,13 activate the receptor for platelet-activating factor (PAF) on leukocytes at submicromolar concentrations and thereby induce leukocyte adhesion to gelatin-coated plastic. Since these fragmented phospholipids are degraded by PAF acetylhydrolase14 and since they activate leukocytes in a fashion that can be inhibited by specific PAF receptor antagonists,12,13 they are now referred to as PAF-like lipids (PAF-LL; see Reference 13).

Inasmuch as the inhibition of oxLDL-induced leukocyte/endothelium interaction by SOD pointed toward the involvement of oxygen radicals in this event, we assumed that PAF and/or PAF-LL formed on endothelial cell membranes in response to oxLDL could mediate oxLDL-induced leukocyte adhesion. To test this hypothesis in vivo, we investigated by intravital fluorescence microscopy whether pretreatment of hamsters with a specific PAF receptor antagonist would attenuate leukocyte/endothelium interaction after systemic administration of oxLDL.

Methods

Animal Model

For our study we used Syrian golden hamsters (5 to 7 weeks old; weight, 55 to 70 g) that were maintained on...
standard lab chow and water ad libitum. Titanium skinfold chambers and indwelling jugular vein and carotid artery catheters were implanted in pentobarbital-anesthetized hamsters as previously described. A recovery period of 48 to 72 hours between chamber implantation and the experiments was allowed to eliminate the acute effects of anesthesia and surgical trauma on the microvasculature. The study complied with German government guidelines for the care and use of laboratory animals.

**Intravital Fluorescence Microscopy**

Intravital fluorescence microscopy was performed in awake animals as previously described in detail. Leukocytes/endothelium interaction, vessel diameters, and red blood cell velocities were assessed in five postcapillary venules (diameter of 20 to 60 μm) and five arterioles (diameter of 20 to 60 μm) per observation chamber. The identical vessel segments were examined before and over the time course after injection of oxLDL by using a computer-controlled stepping motor-driven platform. Leukocytes were stained in vivo with acridine orange (0.5 mg · kg⁻¹ · min⁻¹ IV) and classified according to their interaction with the endothelial lining as adherent, rolling, or free-flowing cells. In arterioles and postcapillary venules, adherent leukocytes were defined in each vessel segment as those cells that did not move or detach from the endothelial lining within an observation period of 30 seconds and are reported as the number of cells per square millimeter of vessel surface, as calculated from the diameter and length (200 μm) of the vessel segment studied. In postcapillary venules, rolling leukocytes are reported as a percentage of nonadherent leukocytes passing through the observed vessel segment within 30 seconds. In arterioles, the high erythrocyte velocity made it impossible to detect free-flowing leukocytes. Rolling leukocytes are thus reported as those cells slowly passing the arteriolar segment within 30 seconds, normalized for the inner vessel circumference. At all time points before and after injection of oxLDL, vessel diameters (diam) were assessed by means of a computer-assisted microcirculation analysis system (CAMS; Reference 17), and centerline red blood cell velocities (velo) were assessed by dual-slit cross correlation. The venular wall shear rate was calculated as [(velo/1.6) × (1/diam) × 8] and given in s⁻¹.

**Lipoproteins**

Isolation and oxidative modification of LDL were performed as previously described in detail. Briefly, LDL was isolated by density-gradient centrifugation (from EDTA-anticoagulated blood of seven healthy human subjects (20 to 30 years of age). The density cut was d= 1.045 to 1.065 g/mL. LDL stock suspensions were stored (4°C under argon in the dark) for a maximum of 7 days. Before oxidative modification of LDL, EDTA was removed by chromatography on Sephadex columns (PD-10, Sephadex G-25M from Pharmacia Fine Chemicals, Uppsala, Sweden). Cholesterol content was determined by Cholesterin Monotest (Boehringer Mannheim GmbH, Mannheim, FRG). LDL suspension was diluted with phosphate-buffered saline (PBS, without Ca²⁺ and Mg²⁺; pH 7.3 at 21°C) to reach a final concentration of 0.85 mg/mL LDL cholesterol. Oxidative modification was achieved by incubation (18 hours, 37°C) of the LDL suspension (1 to 1.5 mL) in 7.5 μmol/L Cu²⁺ (CuSO4·5H2O; Merck, Darmstadt, FRG). In earlier studies endotoxin contamination of LDL and oxLDL suspensions prepared in this fashion had been ruled out by both the Limulus assay and experiments in which heat-inactivated oxLDL suspensions failed to induce leukocyte adhesion in the same animal model (H.A. Lehr et al, unpublished observations, 1993). Immediately after oxidation, oxLDL (4 mg LDL cholesterol per kilogram of body weight) was injected intravenously as a bolus into the hamsters via the permanent jugular vein catheter. Cu²⁺ was not removed from the oxLDL suspension before injection into the animals. In control experiments we had observed that neither free Cu²⁺ (7.5 μmol/L CuSO4 in PBS) nor a freshly prepared LDL/Cu²⁺ suspension stimulated the interaction of fluorescently stained leukocytes with the microvascular endothelium. LDL oxidation was verified by fatty acid determinations of native LDL and oxLDL as well as by assessment of the electroophoretic mobility of LDL particles on agarose gel.

**PAF Receptor Antagonist**

WEB2170, a selective heterazepinoic antagonist of PAF, was kindly provided by C. Meade, MD, Boehringer Ingelheim, Ingelheim, FRG. Hamsters were pretreated 10 minutes before injection of oxLDL with WEB2170, which was administered intravenously at a dose of 1 mg/kg of body weight in 200 μL normal saline (0.15 mol/L NaCl). Control animals were treated with an equivalent volume of normal saline.

**Statistical Analysis**

Values were tested for parametric distribution. Although parametric distribution was not uniformly found in all data sets, the data in the figures are given as mean±SD to facilitate rapid interpretation. Probability values were calculated using the Mann-Whitney U test or the Wilcoxon test with Bonferroni correction. Values of P<.05 or <.01 were considered significant and are denoted in the figures with one or two asterisks, respectively. Correlations between venular wall shear rates and leukocyte/endothelium interaction were calculated using the Spearman test.

**Results**

**Microcirculatory Changes After Injection of OxLDL**

Under baseline conditions the majority of leukocytes did not interact with either the arteriolar or venular endothelium. Intravenous injection of oxLDL (4 mg LDL cholesterol per kilogram of body weight) immediately induced leukocyte rolling along the endothelial lining and subsequent firm adhesion to the endothelial surface of both arterioles and postcapillary venules (Figs 1 through 3). The higher total number of sticking leukocytes per square millimeter of endothelial surface in postcapillary venules compared with arterioles (Figs 1B and 2B) may be due to differences in local hemodynamic conditions, with low shear rates prevailing in venules and high shear forces in arterioles. Furthermore, differences in endothelial cell phenotype between arterioles and venules may have contributed to this discrepancy.

Both rolling and sticking leukocytes tended to form aggregates of three cells or more. Although the fluorescence...
cent marker acridine orange does not stain platelets, the loose consistency of the leukocyte aggregates suggests that platelets were involved in their formation.

When injected into WEB2170-pretreated hamsters, oxLDL elicited an increase in the fraction of rolling leukocytes in postcapillary venules that was significantly less pronounced than in untreated animals (Fig 2A). OxLDL-induced leukocyte rolling was almost entirely prevented in arterioles (Fig 1A). Likewise, the firm adhesion of leukocytes to the endothelium was significantly attenuated in both arterioles and postcapillary venules in animals pretreated with WEB2170 (Figs 1B and 2B). The extent to which WEB2170 inhibited oxLDL-induced leukocyte/endothelium interaction is comparable to the effects seen in animals pretreated with leukotriene inhibitors* and SOD. The occurrence of loose leukocyte aggregates in response to oxLDL injection was no longer observed. Neither control nor WEB2170-treated animals showed significant changes in red blood cell velocity and vessel diameter in postcapillary venules after injection of oxLDL (not shown). In particular, no difference in microhemodynamic parameters was observed between WEB2170-treated and control animals. Both the increase in leukocyte rolling and sticking to the venular endothelium in response to oxLDL and the inhibition of this event by WEB2170 pretreatment were not due to alterations in local hemodynamic and, hence, wall shear conditions (Fig 3). It should be noted that under the low shear rates prevailing in the venular segments in striated muscle, no correlation was found between wall shear rate and leukocyte/endothelium interaction, neither at baseline

FIG 1. Bar graphs of leukocyte/endothelium interaction in arterioles after injection of oxidized low-density lipoprotein (oxLDL). Leukocyte rolling (panel A) and adhesion (panel B) were assessed in five arterioles in each observation chamber before injection of oxLDL and during the time course thereafter. For contrast enhancement, leukocytes were stained in vivo with acridine orange. Measurements were performed on control hamsters (n=7, open bars) and hamsters pretreated with WEB2170 (1 mg/kg of body weight IV, 10 minutes before injection of oxLDL; n=7, black bars). Rolling leukocytes are defined as cells slowly traversing the observed vessel segment within 30 seconds, expressed in proportion to the inner vessel circumference. Adherent leukocytes are expressed as the number of cells per square millimeter of endothelial surface. Data given in the figure are mean±SD. *P<.05, **P<.01 versus corresponding values in control animals (Wilcoxon test with Bonferroni correction).

FIG 2. Bar graphs of leukocyte/endothelium interaction in postcapillary venules after injection of oxidized low-density lipoprotein (oxLDL). Leukocyte rolling (panel A) and adhesion (panel B) were assessed in five postcapillary venules in each observation chamber before injection of oxLDL and during the time course thereafter. For contrast enhancement, leukocytes were stained in vivo with acridine orange. Measurements were performed on control hamsters (n=7, open bars) and hamsters pretreated with WEB2170 (1 mg/kg of body weight IV, 10 minutes before injection of oxLDL; n=7, black bars). Rolling leukocytes are expressed as the percentage of all nonadherent cells passing through the observed vessel segment within 30 seconds. Adherent leukocytes are expressed as the number of cells per square millimeter of endothelial surface. Data given in the figure are mean±SD. *P<.05, **P<.01 versus corresponding values in control animals (Wilcoxon test with Bonferroni correction).
FIG 3. Plots showing interrelations between venular wall shear rate and leukocyte/endothelium interaction. Leukocyte rolling (panel A) and adhesion (panel B) were assessed in five postcapillary venules in each observation chamber before injection of oxidized low-density lipoprotein (oxLDL) and during the time course thereafter. For contrast enhancement, leukocytes were stained in vivo with acridine orange. Measurements were performed on control hamsters (n=7, left panels) and hamsters pretreated with WEB2170 (1 mg/kg of body weight IV, 10 minutes before injection of oxLDL; n=7, right panels). Rolling leukocytes are expressed as a percentage of all nonadherent cells passing through the observed vessel segment within 30 seconds [%]. Adherent leukocytes are expressed as the number of cells per square millimeter of endothelial surface [I/mm²]. Centerline red blood cell velocity (velo) was assessed by dual-slit cross correlation, and microvessel diameter (diam) was assessed by CAMAS. Wall shear rates were calculated as [(velo)1.6] x (1/diam) x8 and are expressed as 1/sec. Correlations between shear rates and leukocyte/endothelium interaction were calculated using the Spearman test. R values never exceeded 0.5.

Discussion

Leukocyte adhesion at sites of enhanced vascular permeability is a constant finding in long-term cholesterol-feeding studies in various laboratory animals as well as in human atherosclerotic lesions (reviewed in Reference 21). In vitro studies have linked the adhesion of leukocytes to the action of oxLDL.54 Using the dorsal-skinfold chamber model and intravital fluorescence microscopy, we have previously demonstrated that systemic administration of oxLDL stimulates leukocyte/endothelium interaction in vivo.8,11 We demonstrate in the present study that oxLDL-induced leukocyte/endothelium interaction in vivo can be significantly attenuated by pretreatment of hamsters with a specific PAF receptor antagonist, WEB2170 (Figs 1 through 3). The extent of inhibition of leukocyte adhesion by WEB2170 corresponds well with the reduction of leukocyte adhesion by other PAF receptor antagonists observed in in vitro experiments.22 Neither oxLDL-induced leukocyte adhesion nor the inhibition of this event by WEB2170 can be ascribed to changes in microhemodynamic parameters and, hence, changes in local shear conditions, since wall shear rates were not significantly different between control and WEB2170-treated animals (Fig 3). The observation that, under baseline and also under any experimental condition, the extent of leukocyte/endothelium interaction did not correlate with alterations in local wall shear rates (Fig 3) is in agreement with studies performed on mesenteric vessels in rats23 and rabbits.24
While pretreatment of the animals with the PAF receptor antagonist almost entirely prevented firm leukocyte adhesion, the effect on leukocyte rolling along the venular endothelium was considerably less pronounced. This finding is in agreement with a study on the cat mesentery, in which PAF receptor antagonists were reported to significantly attenuate posts ischemic leukocyte adhesion while exerting only minimal effects on leukocyte rolling. We presume that this discrepancy may reflect differences in receptor molecules involved in leukocyte rolling (selectins) and leukocyte adhesion (β₂-integrins; reviewed in Reference 26).

Unstimulated polymorphonuclear leukocytes and monocytes express on their surface a receptor for PAF, which is a potent activator for chemotaxis and adhesion to endothelial cells. In a complex system of juxtacrine cell-cell interaction, it was shown that binding of endothelial cell-confined PAF and/or PAF-LL to the PAF receptor stimulates leukocyte adhesion through an upregulation of the CD11b/CD18 adhesion receptor complex on these cells. The time course of PAF presentation on endothelial cells in response to endothelial activation as well as the time course of PAF-induced upregulation of CD11b/CD18 on leukocytes and adhesion of leukocytes to cultured endothelial cells corresponds well with the response time of oxLDL-induced leukocyte/endothelium interaction observed in our experiments (Figs. 1 and 2). The results of this study may suggest that oxLDL stimulates in endothelial cells the enzymatic generation of PAF from a phospholipid precursor and its subsequent translocation to the endothelial cell surface. However, the inhibition of oxLDL-induced leukocyte/endothelium interaction by the specific PAF receptor antagonist does not necessarily imply the generation and action of authentic PAF, since numerous other fragmented phospholipids likewise bind to and activate this receptor. These fragmented phospholipids, referred to as PAF-LL, generated through uncontrolled oxygen radical-catalyzed peroxidation of membrane phospholipids and have been found to induce leukocyte adhesion in vitro. Furthermore, it is conceivable that oxLDL could exert PAF-like activity per se. This notion is based on the structural similarities between oxLDL and PAF. LDL is associated with a modified phospholipids and thus, to protect the organism from the effects of oxidized LDL.

The demonstration that oxLDL-induced leukocyte/endothelium interaction involves the action of PAF and/or PAF-LL follows up on a series of experiments performed in the same animal model. In those studies it was shown that under identical experimental conditions oxLDL-induced leukocyte adhesion can be attenuated to a comparable extent by SOD and by a specific inhibitor of leukotriene biosynthesis. These observations are in agreement and exceed the earlier findings demonstrating that SOD, inhibitors of leukotriene biosynthesis or action, and PAF receptor antagonists attenuate leukocyte/endothelium interaction in response to diverse pathophysiological conditions, such as acute or chronic inflammation and ischemia/reperfusion. These studies suggest that, irrespective of the initial pathophysiological stimulus, leukocyte/endothelium interaction involves the generation and action of oxygen radicals, leukotrienes, and receptors for PAF and/or PAF-LL.

Elaborate in vitro studies have demonstrated a complex network of amplification interrelations between oxygen radicals, leukotrienes, and PAF and/or PAF-LL. In agreement with these studies, we propose that injection of oxLDL into the hamster organism induces the radical-dependent peroxidation of membrane phospholipids and the generation of PAF or PAF-LL, which then bind to PAF receptors on the leukocyte surface and stimulate leukocyte adhesion through a leukotriene-dependent mechanism. Through further release of oxygen radicals, leukotrienes, PAF, and other inflammatory mediators, adherent leukocytes could then contribute to the recruitment and adhesion of additional leukocytes, even when oxLDL, the initial stimulus, has been cleared from the circulation. The observations of the present and previous studies that inhibition of either oxygen radicals, leukotrienes, or the PAF receptor will significantly attenuate oxLDL-induced leukocyte adhesion may help to understand the mechanism of action by which antioxidants, PAF receptor antagonists, and leukotriene inhibition through dietary fish oil exert their protective effects in animal models of diet-induced atherogenesis.

Acknowledgments

This study was supported in part by the Deutsche Forschungsgemeinschaft through Sonderforschungsbereich 320, by research grant Hu 408/1-4, and by a grant from the Transplantations schwerpunkt Heidelberg. The excellent technical assistance of B. Schringer-Mansour and I. Wernicke is gratefully acknowledged. We appreciate the generosity of Dr. T.M. McIntyre (Nora Eccles Harrison Cardiovascular Research and Training Institute, Salt Lake City, Utah) and Dr. U. von Andrian (La Jolla Institute for Experimental Medicine, La Jolla, Calif) for making preprints of manuscripts available to us.

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_Arterioscler Thromb Vasc Biol._ 1993;13:1013-1018
doi: 10.1161/01.ATV.13.7.1013

_Arteriosclerosis, Thrombosis, and Vascular Biology_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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