Oxidized Low-Density Lipoprotein Activates Migration and Degranulation of Human Granulocytes

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Oxidized low-density lipoprotein (oxLDL) has been reported as a major participant in the pathogenesis of atherosclerosis. We hypothesized that oxLDL can also interact with granulocytes during inflammatory airway diseases, such as asthma. To test the chemotactic effect of oxLDL, isolated human peripheral granulocytes were added to the upper chambers of Transwell filters and migration in response to oxLDL was determined. Cu^{+2}-oxidized LDL stimulated neutrophil (23.4 ± 3.2% for 100 μg/ml oxLDL versus 2.9 ± 1.1% for buffer, $P < 0.05$) and eosinophil (19.3 ± 3.5% versus 0.6 ± 0.02% for buffer, $P < 0.05$) chemotaxis in a concentration-dependent manner. The magnitude of chemotaxis was dependent on the degree of LDL oxidation. Granulocyte transmigration across IL-1β–activated human pulmonary microvascular endothelial cell monolayers was similarly stimulated by oxLDL. OxLDL activated significant degranulation of both neutrophils (100.9 ± 9.8 versus 49.6 ± 8.4 ng lactoferrin released/5 × 10^5 neutrophils for buffer, $P < 0.05$) and eosinophils (342 ± 115.4 versus 85.8 ± 30.4 ng eosinophil-derived neurotoxin/1 × 10^6 eosinophils for buffer, $P < 0.05$). Therefore, *in vivo* influx and oxidation of LDL may be an important mediator for the initiation of bronchial inflammation where granulocytes are recruited to the lung.

In vivo oxidation of native low-density lipoprotein (nLDL) has been proposed to play a crucial role in the initiation and propagation of arteriosclerosis via its effects on peripheral blood monocytes and tissue macrophages (1). Although the initiating event of LDL oxidation in arteriosclerosis is not known, one mechanism of oxidized LDL (oxLDL) participation is in the chemotaxis of monocytes and macrophages to the vascular bed and its conversion of monocytes into macrophages and then into foam cells resulting in plaque deposition and cardiovascular damage (1). However, participation of oxLDL is not confined to cardiac disease; it has been implicated in multiple inflammatory processes, including diabetes and renal disease (1, 2). Although the site of LDL oxidation and leukocyte interaction is the vascular wall in arteriosclerosis, inflamed tissue characterized by vascular permeability and leukocyte infiltration would provide an optimal environment for LDL oxidation. Oxidation of LDL has been suggested as a modulator of asthma inflammation (3).

Although monocyte infiltration and foam cell formation are not features of asthma exacerbations, the profound influx of first neutrophils and then eosinophils to the airway may promote inflammation through their interactions with LDL. Asthma is characterized by increased vascular permeability, which would promote influx of circulating nLDL (4). Once in the airway tissue, multiple *in vivo* inflammatory mediators of asthma are capable of modulating LDL oxidation including nitric oxide, inducible nitric oxide synthase, mast cells, macrophages, and leukocytes capable of generating high levels of toxic oxygen metabolites (neutrophils, eosinophils, and monocytes) (5). In turn, peroxidation of the multiple lipids in LDL may generate key inflammatory mediators of granulocyte recruitment and activation and, hence, promote bronchial hyperresponsiveness (3). Activation of the neutrophil's respiratory burst resulting in the generation of multiple potent oxygen metabolites and release of granulocyte peroxidases can promote LDL oxidization (6). OxLDL can, in turn, stimulate the neutrophil's respiratory burst (7) and promote cell adhesion (8).

We hypothesized that oxLDL is an inflammation mediator which can recruit and activate granulocytes. Reports on the effects of oxLDL on the function of circulating neutrophils, and, especially, eosinophils, are very limited, and comparisons with monocytes have not been reported. In this study, human peripheral blood neutrophils and eosinophils were isolated and their response to nLDL and oxLDL was measured in relationship to chemotaxis, transendothelial migration, degranulation, and expression of LDL receptors (LDLRs).

Materials and Methods

Reagents

The following reagents were obtained as noted: Hanks Balanced Salt Solution (HBSS), fetal calf serum (FCS), and Roswell Park Medical Institution (RPMI) from Life Technologies (Rockville, MD); Percoll from Amersham/Pharmacia (Piscataway, NJ); BODIPY-labeled LDL (Intracel, Frederick, MD) (555 nm excitation/571 nm emission). Anti-LDLR monoclonal antibodies (mAbs) were purchased as follows: anti-LDLR (LDLR: clone C7) from Amersham/Pharmacia; anti-CD36 (clone FA6–152) from Immunotech (Miami, FL); anti-CD68 (clone Y1/82A), mouse isotype controls immunoglobulin (Ig) $G1_1$ and IgG_2, and goat anti-mouse IgG–fluorescein isothiocyanate

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Abbreviations: absorbance at 234 nm (Abs_{234}); cytochalasin B, CB; eosinophil-derived neurotoxin, EDN; ethylenediaminetetraacetate, EDTA; fluorescence activated cell sorting, FACS; fetal calf serum, FCS; granulocyte-macrophage colony-stimulating factor, GM-CSF; Hanks balanced salt solution, HBSS; human pulmonary microvascular endothelial cells, HPMEC; immunoglobulin, Ig; low-density lipoprotein, LDL; LDL receptor, LDLR; lactoferrin, LTF; native LDL, nLDL; monoclonal antibody, mAb; mean fluorescence units, MFU; oxidized LDL, oxLDL; platelet activating factor, PAF; percent positively labeled cells, %POS; red blood cells, RBC.

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from BD/Pharmingen (San Diego, CA). All antibodies were titered to determine optimal binding concentrations. All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

LDL Oxidation

Human plasma LDL was purchased from Intracel (Frederick, MD) and the presence of a single band confirmed by agarose gel electrophoresis. After dialysis (4°C, 48 h, with a 30-kD molecular weight cutoff) to remove ethylenediaminetetraacetate (EDTA), nLDL (2.5 mg/ml) was oxidized with 5 μM $CuSO_4$ for 20–24 h at 30°C and dialysis was then repeated to remove the $Cu^{+2,3}$ (9). The level of peroxidation was confirmed by the formation of conjugated dienes and measured by absorbance at 234 nm (Abs_{234}). Alternatively, nLDL was spontaneously oxidized after dialysis of EDTA by exposure to natural light and air. The oxidation level was monitored daily by Abs_{234}. In studies on the effect of nLDL, EDTA was dialyzed out immediately before use to minimize any oxidation; only LDL with $Abs_{234} < 0.3$ was used as nLDL.

Human Subjects

Leukocytes were isolated from peripheral blood of subjects with allergic rhinitis or mild allergic asthma. Subjects ranged in age from 18–55 yr and sex distribution was equal. Immediate hypersensitivity was confirmed, using the prick-puncture technique, by at least one positive skin reaction (> 3 mm) to extracts of common allergens including ragweed, house dust mite, grass pollen, cat dander, and dog dander. Except for use of inhaled β_2-agonists as needed, subjects were taking no medications at the time of study and no corticosteroids within 6 mo. Informed, written consent was obtained from all subjects before their participation in the study, which was approved by the University of Wisconsin Human Subjects Committee.

Leukocyte Isolation

Neutrophils. Heparinized venous blood was diluted with an equal volume of HBSS without Ca^{+2} (HBSS−Ca), and density fractionated over 1.077 g/ml Percoll (20 min at 700 × *g*). The mononuclear cell band at the plasma/Percoll interface was removed (*see below*) and the granulocyte/red blood cells (RBC) pellets collected. The RBCs were removed by hypotonic lysis and the resulting neutrophils were > 95% pure and > 98% viable by trypan blue exclusion. Contaminating cells were eosinophils.

Eosinophils. Peripheral blood eosinophils were isolated using negative immunomagnetic bead selection (10). Briefly, heparinized venous blood was density fractionated over 1.090 g/ml Percoll. After RBC lysis, the resulting granulocytes were resuspended with mouse anti-human CD16-labeled magnetic beads (Miltenyi Biotechnology, Inc., Auburn, CA) for 40 min at 4°C. The cells were then passed through a magnetic field (AutoMacs; Miltenyi Biotechnology, Inc.) and CD16-negative eosinophils were collected. Eosinophils were > 97% pure and > 98% viable. Contaminating cells were neutrophils and mononuclear cells.

Mononuclear cells. After density fractionation over Percoll, the peripheral blood mononuclear cell band at the HBSS − Ca^{2+}/Percoll interface was washed twice in HBSS − Ca. For LDLR experiments, no further cell isolation was done and the monocytes and lymphocytes were individually gated by fluorescence activated cell sorting (FACS) (*see below*). For chemotaxis experiments, monocytes were further purified using positive selection with anti-CD14–labeled magnetic beads (Miltenyi) similar to the eosinophil purification given above. The monocytes were > 90% pure (contaminating cells were lymphocytes) and > 98% viable.

Endothelial cell culture

Human pulmonary microvascular endothelial cells (HPMEC) cryopreserved as tertiary or quaternary cultures were purchased from BioWhittaker (San Diego, CA) (11). HPMEC were characterized as endothelial cells by acetylated LDL uptake, factor VIII–related antigen expression, and positive staining for platelet endothelial cell adhesion molecule 1 (CD31). Endothelial cell growth medium 2MV, supplemented with 10 ng/ml human recombinant epidermal growth factor, 1 μg/ml hydrocortisone, 50 μg/ml gentamycin, 50 ng/ml amphotericin-B, 12 μg/ml bovine brain extract, and 5% FCS, was obtained from BioWhittaker. To promote HPMEC attachment and growth, all culture surfaces were precoated with 10 μg/ml plasma fibronectin for 1 h at 37°C. HPMEC were passaged before they reached 90% confluence. Endothelial cells, derived from several different donors, were used at passages 4 through 8 and found to give equivalent results.

Chemotaxis

Eosinophils, neutrophils, or monocytes (3–5 × 10^6 cells/ml) in HBSS + 0.1% gelatin (HBSS/gel) were added to the upper compartment of Transwell 24-well plates (Costar, Cambridge, MA) with 3 μm (neutrophils and monocytes) or 5 μm (eosinophils) polycarbonate membrane filters (10). The different pore sizes were used to optimize the chemoattractant-stimulated migration while minimizing negative control (HBSS/gel only) migration. HBSS/gel with or without LDL was added to the lower compartment; 10 nM platelet activating factor (PAF) or n-formyl methionyl, leucyl, phenylalanine (FMLP) were used as positive controls. After a 1-h incubation in 5% CO_2 at 37°C, 25 mM EDTA was added to the bottom wells for 5 min to release any cells that were adhered to the underside of the filter. Cell samples from the bottom wells were counted in quadruplicate by hemacytometer (Neubauer hemacytometer, Hausser Scientific, Horsham, PA) and percent chemotaxis was determined as the number of cells migrated to the lower chamber divided by the total number of cells added to the upper chamber × 100. To determine if oxLDL was chemokinetic rather than chemotactic for granulocytes, 100 μg/ml oxLDL was added to the top Transwell chamber only, to the bottom chamber only, or to both chambers, and migration of the granulocytes was then measured.

Transendothelial Migration

HPMEC (2.5 × 10^5 cells/ml) were cultured on plasma fibronectin (10 μg/ml)–coated Transwell inserts. Endothelial cell medium was added only to the upper Transwell chamber to inhibit the formation of an HPMEC bilayer; monolayers formed within 2 d and were confirmed for confluence by transendothelial electrical resistance (12) and cobblestone morphology (Diff-Quik; Baxter Scientific Products, McGraw Park, IL). Confluent monolayers were stimulated with IL-1β (100 pM, 6 h; R&D Systems, Minneapolis, MN) to enhance intracellular adhesion molecule 1 expression on HPMEC and, hence, enhance granulocyte migration (10). Following cytokine treatment, the monolayers were washed three times with 37°C HBSS. Eosinophils (8-μm pore filters; 3.5–5 × 10^6 cells/ml) or neutrophils (3-μm pore filters) in culture medium (RPMI supplemented with 5% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin) were then added to the upper chamber. Culture medium only or with 10 nM PAF or LDL, was added to the lower chamber. After a 3-h incubation in 5% CO_2 at 37°C, 25 mM EDTA was added to the bottom wells and percent cell migration was determined as for chemotaxis.

Degranulation

Neutrophils (5×10^5/ml) were activated with degranulation buffer (HBSS + 0.03% gelatin), FMLP (100nM), PAF (100nM), granulocyte-macrophage colony-stimulating factor (GM-CSF) (1 ng/ml), or oxLDL (100 μg/ml) in the presence of 5 μg/ml cytochalasin B (CB) for 15 min at 37°C. The samples were cooled on ice and the cell-free supernatants were stored at −20°C until quantitated for released lactoferrin (LTF) by commercial enzyme-linked immunosorbent assay according to manufacturer's directions (Oxis Research, Portland, OR).

Eosinophils (1×10^6/ml) were activated with degranulation buffer, FMLP (100 nM), IL-5 (1 ng/ml), GM-CSF (1ng/ml), or oxLDL (100 μg/ml) for 4 h at 37°C in a 5% CO_2 incubator. The cell-free supernatants were stored at −20°C until assayed for released eosinophil-derived neurotoxin (EDN) by radioimmunoassay (RIA) (13).

Leukocyte LDLR

To determine specific binding of LDL, isolated leukocyte populations were incubated for 30 min at 4°C with 5 μg/ml BODIPY-labeled LDL in the absence or presence of unlabeled 50 μg/ml LDL, washed, and the percentage of BODIPY-positive cells was determined by FACS. To identify specific leukocyte LDLR, peripheral blood eosinophils, neutrophils, or mononuclear cells (1×10^5/100 μl) were incubated in FACS buffer (phosphate-buffered saline + 2% bovine serum albumin + 0.2% sodium azide) at 4°C with mouse anti-human LDLR (clone C7), anti-CD36, anti-CD68 mAbs, or corresponding isotype controls. After a wash with FACS buffer, fluorescein isothiocyanate–labeled goat anti-mouse IgG was added for 30 min. Using FACS (FACScan, Becton Dickinson), gates for each cell type were defined, 10,000 events collected, and the mean fluorescence units and percentage of positively labeled cells were determined using CellQuest software (Becton Dickinson). Isotype control values were subtracted from the reported values for each specific receptor.

Statistical Analysis

Data are presented as the mean ± SEM and were analyzed by analysis of variance (SigmaStat; SPSS, Chicago, IL). When significant differences were found within the data groups, individual differences were then analyzed by paired *t* test. A *P* value < 0.05 was considered significant.

Results

Chemotaxis

LDL oxidation was confirmed by monitoring conjugated diene formation at Abs_{234} (9). To determine the chemotactic effect of nLDL ($Abs_{234} < 0.3$) versus Cu^{2+}-oxidized LDL ($Abs_{234} > 0.75$), increasing concentrations of lipid were placed in the bottom chambers of Transwell plates and neutrophils or eosinophils were placed in the upper chamber. OxLDL dose-dependent migration occurred with neutrophils and was significant at 10 μg/ml (Figure 1A). OxLDL stimulated eosinophil migration only at the 100 μg/ml concentration (Figure 1B). As positive controls, 10 nM PAF activated 25.2 ± 1.6% eosinophil chemotaxis and 10 nM FMLP stimulated 30.2 ± 1.4% neutrophil chemotaxis; these levels were comparable to the effects of 100 μg/ml oxLDL. Neither granulocyte population underwent migration with any concentration of nLDL, and increasing oxLDL to 500 μg/ml did not further enhance leukocyte chemotaxis.

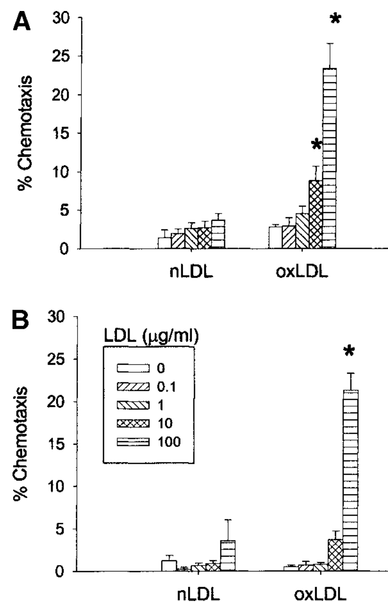

Figure 1. Effect of oxidized low-density lipoprotein (oxLDL) on granulocyte chemotaxis. Neutrophils (*A*) or eosinophils (*B*) were added to the top of Transwell filters and increasing concentrations of either native LDL (nLDL) ($Abs_{234} < 0.3$) or oxLDL ($Abs_{234} > 0.75$) were added to the bottom chambers. After a 1-h incubation, the number of migrated cells were counted. Mean ± SEM of 10 individual experiments each performed in duplicate; *$P < 0.05$ versus no LDL.

To determine the relative effect of partially oxLDL on granulocyte chemotaxis, nLDL was dialyzed to remove EDTA and then allowed to spontaneously oxidize by exposure to natural light and air. At 100 μg/ml, nLDL ($Abs_{234} < 0.3$) had no significant effect on granulocyte chemotaxis (Figure 2). As the oxidative level of the LDL increased, so did the levels of neutrophil and eosinophil chemotaxis until a maximum at $Abs_{234} = 0.6–0.7$ (> 50% migration). How-

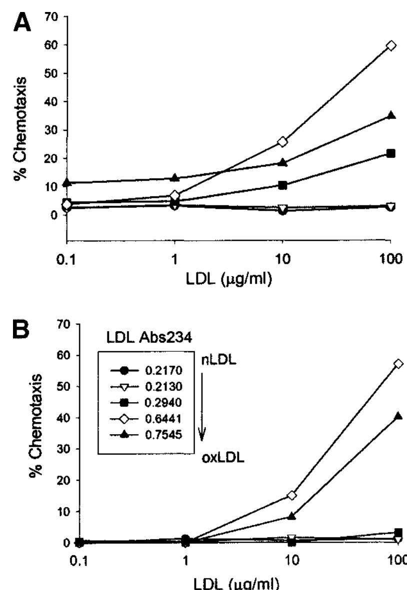

Figure 2. Level of LDL oxidation determines granulocyte chemotaxis. LDL was oxidized by exposure to natural light and air over time. 100 μg/ml oxLDL at increasing oxidation levels was used as the chemoattractant for neutrophils (*A*) or eosinophils (*B*). *n* = two experiments done in duplicate.

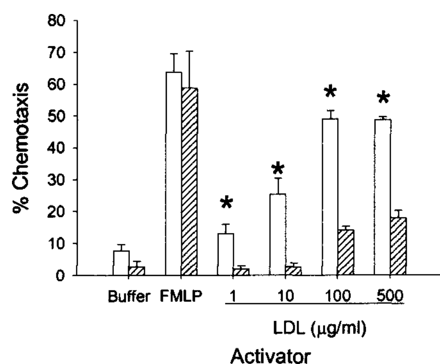

Figure 3. Comparison of neutrophil (*open bars*) and monocyte (*dashed bars*) chemotaxis to oxLDL (Abs_{234} = 0.6–0.7). The two-cell populations were equivalent in chemotaxis for the negative (Hanks' Balanced Salt Solution [HBSS]/gel only) and positive (10 nM n-formyl methionyl leucyl phenylalanine [FMLP]) controls. Neutrophil chemotaxis was significantly increased over monocyte chemotaxis at all concentrations of oxLDL. Mean + SEM of three experiments performed in duplicate; $*P < 0.05$ for neutrophils versus monocytes.

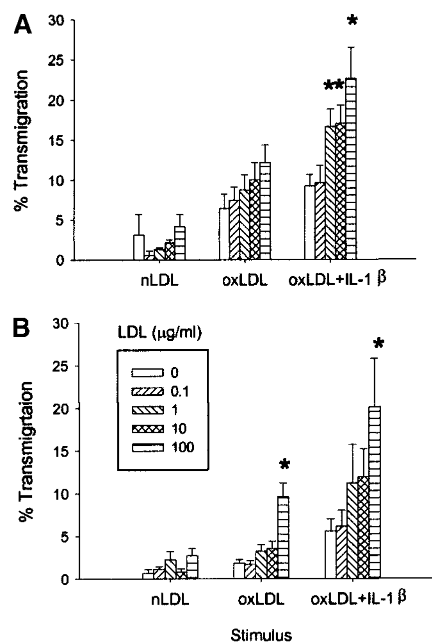

Figure 4. Transendothelial migration of neutrophils (*A*) or eosinophils (*B*) across human pulmonary microvascular endothelial cells (HPMEC) monolayers in response to native LDL (nLDL) ($Abs_{234} < 0.3$), oxLDL ($Abs_{234} > 0.75$; $CuSO_4$ oxidized), or oxLDL with interleukin (IL)-1β pretreated (6 h, 100 pM) HPMEC. Granulocytes were added to confluent HPMEC monolayers while oxLDL was placed in the bottom chamber. After a 3-h incubation, the migrated cells in the lower chamber were counted. Mean ± SEM of five experiments each done in duplicate; $*P < 0.05$ versus no LDL.

ever, with extensive spontaneous oxidation ($Abs_{234} > 0.75$), granulocyte chemotaxis actually decreased to levels observed with Cu^{+2}-oxidation (Figure 1).

To optimize chemotaxis and compare the responses of circulating neutrophils and monocytes, increasing concentrations of spontaneously oxLDL (Abs_{234} = 0.6–0.7) were used as the chemoattractant (Figure 3). Although the two-cell populations were equivalent in their response to HBSS/gel alone and to 10 nM FMLP, neutrophils demonstrated significantly increased chemotaxis compared with monocytes at all of the oxLDL concentrations evaluated.

To determine if the effect of oxLDL on granulocyte migration was a combination of chemokinesis and chemotaxis, 100 μg/ml of spontaneously oxLDL (Abs_{234} = 0.6–0.7) was added to the top, bottom, or both Transwell chambers, and migration was measured. Addition of oxLDL to the upper chamber resulted in 17 ± 8.1% eosinophil migration compared with 53.4 ± 10.9%, or 17.8 ± 5.7% neutrophil migration versus 50.4 ± 17.3%, when oxLDL was added to the bottom chamber alone. In contrast, granulocyte migration in response to PAF (5.4 ± 4% [PAF upper chamber] versus 36.3 ± 20.6% [PAF in the lower chamber] migration for eosinophils) or FMLP (5.6 ± 2.1% versus 36.1 ± 12.8% migration for neutrophils) had a smaller chemokinetic effect. nLDL demonstrated no migratory effect when added to either chamber.

Transmigration

To determine the effect of oxLDL on granulocyte transendothelial migration, HPMEC monolayers were grown to confluence on Transwell filters. Neutrophils demonstrated increased migration with increasing oxLDL concentrations ($Abs_{234} > 0.75$ by $CuSO_4$ oxidation), but these changes did not reach significance (Figure 4A). However, when HPMEC were pretreated with 100 pM IL-1β for 6 h, neutrophil transmigration was enhanced at concentrations of 1–100 μg/ml oxLDL. In contrast, oxLDL stimulated significant eosinophil migration across both untreated and IL-1β–treated HPMEC monolayers (Figure 4B). As observed with chemotaxis, nLDL did not stimulate neutrophil or eosinophil migration through unactivated or IL-1β activated endothelial monolayers (data not shown).

Degranulation

OxLDL activated both neutrophil and eosinophil degranulation. Neutrophil degranulation stimulated by 100 μg/ml oxLDL (Abs_{234} = 0.6–0.7) resulted in significantly higher levels of LTF release than buffer or GM-CSF, and was comparable to levels activated by FMLP (Figure 5A). The addition of CB (5 μg/ml) was required for neutrophil degranulation. Eosinophil EDN degranulation was significantly activated by oxLDL, FMLP, IL-5, and GM-CSF compared with HBSS/gel alone (Figure 5B). In contrast to its effect on neutrophils, CB inhibited eosinophil degranulation (data not shown). Increasing the oxLDL concentration to 500 μg/ml did not result in additional degranulation and nLDL had no effect over buffer levels on LTF or EDN release (data not shown).

LDLRs

The presence of LDLRs on granulocytes was confirmed by FACS using direct binding of LDL fluorescence-labeled with BODIPY. Neutrophils (73.8 ± 16.5%) and eosinophils

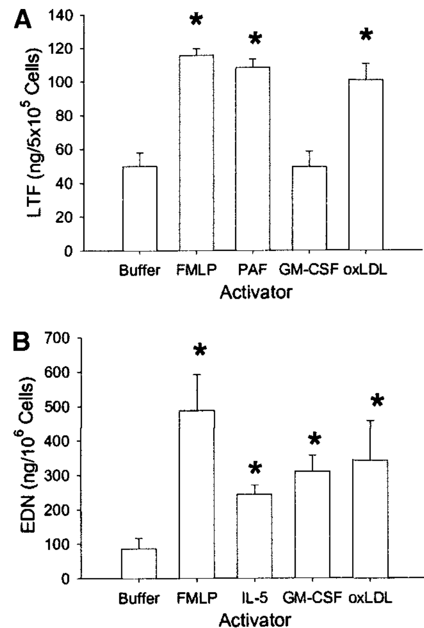

Figure 5. OxLDL degranulation of neutrophils and eosinophils. Neutrophil lactoferrin (LTF) release (*A*) or eosinophil eosinophil-derived neurotoxin (EDN) release (*B*) was activated with FMLP (100 nM), platelet activating factor (PAF; 100 nM), granulocyte-macrophage colony-stimulating factor (GM-CSF; 1 ng/ml), IL-5 (1 ng/ml) or oxLDL (100 μg/ml; $Abs_{234} = 0.6$–0.7). $n = 9$–13 experiments; $*P < 0.05$ versus Buffer.

(70 ± 12.4%) bound BODIPY-labeled LDL. To determine if neutrophils, eosinophils, and monocytes expressed specific LDLRs, each cell population was incubated with anti-LDLR mAbs. Only monocytes expressed a high level of the scavenger receptor CD36, as measured by both mean fluorescence units and percent positive cells, compared with neutrophils and eosinophils (Table 1). In contrast, only neutrophils expressed a significant, albeit low, level of receptor binding to anti-LDLR (C7). Neutrophils, eosinophils, and monocytes all expressed similar, very low levels of CD68.

TABLE 1
*Low-density lipoprotein receptor expression**

Characteristic	Neutrophils	Eosinophils	Monocytes
MFU[‡]			
LDLR (C7)	2.5 + 0.8[†]	0.1 ± 0.8	0
CD68	10.6 ± 5.8	7.5 ± 3.2	6.8 ± 2.1
CD36	0.8 ± 0.9	4.2 ± 2.3	235 ± 40.5[†]
%POS[‡]			
LDLR (C7)	6.1 ± 3[†]	0.12 ± 0.23	0
CD68	1.2 ± 0.4	0.37 ± 0.26	0.25 ± 1.8
CD36	3.3 ± 1.8	4.6 ± 1.8	57.1 ± 11.9[†]

Definition of abbreviations: LDLR, low-density lipoprotein receptor; MFU, mean fluorescence units; %POS, percent positively labeled cells.
* Minus corresponding isotype control value.
[†] $n = 3$.
[‡] $P < 0.05$ versus the other cell types.

PAF-like lipids are constituents of oxLDL and have been implicated in LDL's cellular effects via binding to cell surface PAF receptors (14). To determine if the PAF receptor was utilized in oxLDL chemotaxis of granulocytes, the PAF antagonist WEB 2086 (100 μM; a generous gift from Boehringer Ingelheim Pharmaceutical, Ridgefield, CT) was incubated with neutrophils or eosinophils for 15 min before the addition of 10 nM PAF or 100 μg/ml oxLDL into the bottom Transwell chamber for chemotaxis. WEB 2086 completely inhibited granulocyte chemotaxis to PAF (Figure 6). In contrast, WEB 2086 had no effect on oxLDL-activated chemotaxis for either neutrophils or eosinophils.

Discussion

Postulated as a mechanism for the recruitment and participation of circulating monocytes in the arterial lesions characteristic of atherosclerosis, oxLDL has been reported to be chemotactic for monocytes (15) and to enhance adhesion of these cells to endothelium (16). However, oxLDL may also play a role in airway inflammation during exacerbations of asthma through its effects on granulocytes. We now report that spontaneous and Cu^{2+}-oxLDL are chemotactic for both neutrophils and eosinophils to a greater degree than they are for monocytes. OxLDL also promoted neutrophil and eosinophil transmigration across confluent HPMEC monolayers, especially after endothelial cell activation by IL-1β. Migration was dependent on both LDL concentration and level of oxidation; highly oxidated LDL ($Abs_{234} > 0.75$) became inhibitory to cell migration and survival (data not shown). Moreover, LDL, which was highly oxidized by $CuSO_4$ ($Abs_{234} > 0.75$), activated granulocyte migration comparable to other granulocyte chemokines, FMLP and PAF, whereas, spontaneously oxidized LDL ($Abs_{234} = 0.6$–0.7) exceeded these chemokines. None of these effects on granulocyte migration were observed with nLDL. Finally, the oxLD concentrations required to stimulate granulocyte chemotaxis and degranulation in our study are comparable to those reported to stimulate monocyte/macrophage function and neutrophil respiratory burst (10–500 μg/ml) (17, 18).

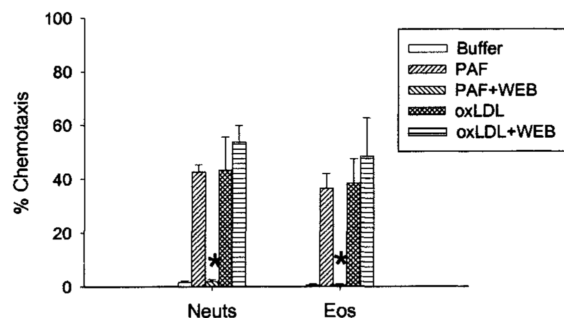

Figure 6. Effect of a PAF receptor antagonist on granulocyte chemotaxis. Preincubation of neutrophils or eosinophils with 100 μM WEB 2086 inhibited 10 nM PAF but not 100 μg/ml oxLDL stimulated chemotaxis. $n = 3$ experiments done in duplicate; $*P < 0.05$ versus PAF alone.

OxLDL (Abs_{234} = 0.6–0.7) also stimulated granule protein release by both granulocyte populations. Neutrophil degranulation of LTF was significantly and similarly activated by oxLDL, FMLP, and PAF, while eosinophil EDN degranulation was stimulated equally by oxLDL, FMLP, IL-5, and GM-CSF. EDN release was used as a measure of eosinophil degranulation because it has relatively low membrane adherence and, hence, good recovery compared with the other three basic granule proteins that characterize eosinophil morphology, major basic protein, eosinophil peroxidase, and eosinophil cationic protein. Interestingly, GM-CSF had no effect on neutrophil LTF degranulation in contrast to its potent effect on eosinophil EDN release. Moreover, the presence of CB was necessary for stimulated LTF degranulation by FMLP, PAF, and oxLDL, but actually inhibited eosinophil degranulation (data not shown), demonstrating a mechanistic difference in the degranulation of these two granulocyte populations. We realize that the effects of oxLDL on LTF and EDN degranulation may not be representative of the release of other granular proteins, but these data support oxLDL as a potent *in vivo* agonist of granulocyte activation. A similar release of granulocyte peroxidases and eosinophil major basic protein would provide a mechanism of airway inflammation, hyperresponsiveness, and further LDL peroxidation in exacerbations of asthma.

OxLDL activation of leukocyte functions is dependent on the cell's expression of LDLRs (19). Because LDL is composed of a heterogeneous collection of lipid particles differing in size, density, and chemical composition, LDLRs are also a collection of different cell membrane glucoproteins (20). Leitinger and coworkers (21) have shown that structurally similar oxidized phospholipids identified in minimally oxLDL can have very different effects on endothelial cell interactions with leukocytes, suggesting different receptors and signaling pathways for very similar lipids. Therefore, identifying the LDLR(s) for any given functional response may require identification of the specific LDL component involved. In addition, the specificity of LDLRs can overlap with the different forms of LDL, such as nLDL, oxLDL, and acetylated LDL (22) or specific cell populations (23). Multiple LDLRs have been identified and include macrophage scavenger receptor CD36 for native and oxLDL (24, 25), activated macrophage scavenger receptor CD68 for oxLDL (26), and neutrophil LDLR, defined by clone C7 antibody (27). In our study, we only measured the expression of these three LDLRs. Neutrophil, eosinophils, and monocytes expressed similar but very low levels of CD68 similar to unactivated macrophages (24), while neutrophils and eosinophils expressed low levels of CD36 compared with much higher monocyte expression. Although neutrophils did express significantly more LDLR than monocytes or eosinophils, these levels were very low as measured by both mean fluorescence (MFU) and percentage of positive cells (% POS), however, low levels of LDLR on neutrophils may be influenced by phagocytosis of LDL/LDLR complexes (27). It is very likely that other receptors specific for oxidatively-modified nLDL exist on these leukocyte populations and were not identified in this study.

Frostegard and coworkers (14) have reported that PAF-receptor antagonist WEB 2170 completely inhibited PAF stimulation, and partially inhibited oxLDL stimulation, of TNF-α synthesis by blood cells. Therefore, some of the observed functional effects of oxLDL could have been the result of its PAF-like lipids. To determine if this was a mechanism of oxLDL-induced chemotaxis, the PAF antagonist WEB 2086 was added to the granulocytes, resulting in complete inhibition of PAF-activated granulocyte migration. In contrast, the addition of WEB 2086 to cells activated with oxLDL had no effect on granulocyte chemotaxis. These data suggest that the effects of oxLDL on granulocytes in these studies were unlikely to be via PAF receptors.

The varied effects of oxLDL on different cell functions, as assessed *in vitro*, may be due to the procedures used to isolate and oxidize LDL, and the criteria selected to define oxidatively modified LDL (21). Our experiments on granulocyte chemotaxis and transendothelial migration initially utilized $CuSO_4$ oxLDL (Abs_{234} > 0.75) and resulted in a significant dose-dependent migration of both neutrophils and eosinophils. However, the high LDL oxidation that occurred with Cu+2 was suboptimal for granulocyte chemotaxis (Figure 2) and induced eosinophil death by 8 h, even in the presence of 0.1 ng/ml IL-5, with total cell death by 24 h (data not shown). This finding agrees with reports on the cytotoxicity of highly oxidized LDL on several types of cells (28, 29). When LDL was partially oxidized ($0.6 < Abs_{234} < 0.7$) by exposure to natural air and light, the degree of chemotaxis was increased and there was no effect on eosinophil viability for over 24 h (data not shown). Others have also reported that partially oxLDL is more effective in the modulation of monocyte and macrophage function and is less toxic to these cells (29, 30). Our "spontaneous" method of oxidation can be closely monitored to achieve specific levels of LDL oxidation. However, it must be acknowledged that different lipids may be oxidized spontaneously compared with the Cu^{+2} reaction. Both methods, however, determined LDL oxidation levels by measuring conjugated dienes and demonstrated similar levels of granulocyte chemotaxis based on the same level of oxidation.

It is possible that the observed chemotactic effects of oxLDL were due to the presence of a chemotactic contaminant in the commercial nLDL preparations. However, several lots of nLDL were oxidized for these studies and the level of functional activation was equivalent at the same LDL concentration and oxidation level. Second, LDL highly oxidized by Cu^{+2}, which may introduce possible chemotactic contaminants, stimulated less granulocyte chemotaxis than spontaneously oxLDL. Third, dialysis to remove EDTA from nLDL and $CuSO_4$ from oxLDL would have eliminated low molecular weight (< 30 kD) contaminants that could have been chemotactic for granulocytes. Finally, nLDL and spontaneously oxidized LDL were handled in an identical manner with the exception that oxLDL was exposed to natural light and air. Therefore, any differences between these lipid species would be due to the oxidation process and not chemotactic contaminants.

Inflammatory airway mediators characteristic of asthma can promote influx and/or oxidation of LDL and several of the actions of oxLDL relate to disease manifestations. *In vivo* activation of cutaneous mast cells and release of histamine enhance transendothelial transport of plasma LDL (31). In

turn, oxLDL can cause mast cell degranulation and increased leukocyte rolling, adhesion, and migration (32). Under certain conditions, oxLDL can itself promote vascular permeability and dilation, thus leading to further edema and leukocyte adhesion (33, 34). Leukocyte and endothelial cell adhesion molecules, including CD11/CD18, L-selectin, intracellular adhesion molecule 1, and P-selectin have been implicated in LDL-elicited recruitment, mast cell degranulation, and increased endothelial permeability (34, 35). OxLDL can also stimulate vasoconstriction and inhibit smooth muscle and endothelial-dependent relaxation (36, 37). These actions, combined with the increased vascular permeability reported in asthma (4), strongly support the participation of oxLDL in the airway inflammation characteristic of asthma.

Inflammation in asthma is a multifactorial process that includes the generation of oxidative events. If a patient with asthma also suffers from hyperlipidemia, exacerbations of this disease may be modulated by the oxidation of airway interstitial LDL (3). We propose that early increases in endothelial permeability characteristic of asthma exacerbations (38) may result in an influx of plasma proteins, including nLDL. Our data support the hypothesis that oxidation of LDL leads to granulocyte migration and degranulation. Therefore, one mechanism of airway inflammation in asthma may be through the recruitment and activation of neutrophils and eosinophils by oxLDL. Upon *in vivo* activation, granulocytes can then generate multiple inflammatory mediators capable of oxidizing nLDL (39), which may perpetuate the process resulting in enhanced and prolonged granulocytic airway inflammation and hyperresponsiveness. Further studies are designed to measure the presence of oxLDL in asthma and thus extend the relevance of these observations to the clinical situation.

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