A Modification of Apolipoprotein B Accounts for Most of the Induction of Macrophage Growth by Oxidized Low Density Lipoprotein*

(Received for publication, November 13, 1998, and in revised form, February 4, 1999)

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It has recently been shown that macrophage proliferation occurs during the progression of atherosclerotic lesions and that oxidized low density lipoprotein (LDL) stimulates macrophage growth. Possible mechanisms for this include the interaction of oxidized LDL with integral plasma membrane proteins coupled to signaling pathways, the release of growth factors and autocrine activation of growth factor receptors, or the potentiation of mitogenic signal transduction by a component of oxidized LDL after internalization. The present study was undertaken to further elucidate the mechanisms involved in the growth-stimulating effect of oxidized LDL in macrophages. Only extensively oxidized LDL caused significant growth stimulation, whereas mildly oxidized LDL, native LDL, and acetyl LDL were ineffective. LDL that had been methylated before oxidation (to block lysine derivatization by oxidation products and thereby prevent the formation of a scavenger receptor ligand) did not promote growth, even though extensive lipid peroxidation had occurred. The growth stimulation could not be attributed to lyso-phosphatidylcholine (lyso-PC) because incubation of oxidized LDL with fatty acid-free bovine serum albumin resulted in a 97% decrease in lyso-PC content but only a 20% decrease in mitogenic activity. Similarly, treatment of acetyl LDL with phospholipase A₂ converted more than 90% of the initial content of phosphatidic acid (PA) to lyso-PC, but the phospholipase A₂-treated acetyl LDL was nearly 10-fold less potent than oxidized LDL at stimulating growth. Platelet-activating factor receptor antagonists partly inhibited growth stimulation by oxidized LDL, but platelet-activating factor itself did not induce growth. Digestion of oxidized LDL with phospholipase A₂ resulted in the hydrolysis of PC and oxidized PC but did not attenuate growth induction. Native LDL, treated with autoxidized arachidonic acid under conditions that caused extensive modification of lysine residues by lipid peroxidation products but did not result in oxidation of LDL lipids, was equal to oxidized LDL in potency at stimulating macrophage growth. Albumin modified by arachidonic acid peroxidation products also stimulated growth, demonstrating that LDL lipids are not essential for this effect. These findings suggest that oxidatively modified apolipoprotein B is the main growth-stimulating component of oxidized LDL, but that oxidized phospholipids may play a secondary role.

Macrophage-derived foam cells play an important role in the early stages of atherosclerosis (1). Cultured macrophages have been shown to take up chemically modified low density lipoproteins (LDLs) such as oxidized LDL and acetyl LDL, resulting in foam cell formation (2, 3). Oxidized LDL exhibits many other potentially atherogenic actions in vitro (2), including direct chemoattractant activity for circulating monocytes (4), induction of macrophage chemotactic protein-1 expression (5), and enhancement of the expression of endothelial adhesion molecules VCAM-1 (vascular cell adhesion molecule-1), ICAM-1 (intercellular adhesion molecule 1), and P-selectin (6–9). These effects would tend to increase the number of macrophages within the arterial intima at sites where oxidized LDL was present.

An additional biological action of oxidized LDL that could increase the number of macrophages within the intima is a mitogenic effect on monocytes or macrophages. It is noteworthy that macrophages have been shown to be the predominant cell type expressing proliferating cell nuclear antigen in atherosclerotic lesions, even in lesions containing mostly smooth muscle-derived cells (10, 11). Oxidized LDL has been reported to be mitogenic for cultured mouse peritoneal macrophages and human monocyte-derived macrophages (12–14). In these studies, the mitogenic effect of oxidized LDL was mediated by protein kinase C (15) and was attributed to lysophosphatidylcholine (lyso-PC) that is generated during LDL oxidation through enzymatic hydrolysis of PC by platelet-activating factor (PAF) acetylhydrolase, an enzyme that is normally associated with LDL (16, 17). It has recently been proposed that this stimulation of macrophage growth involves induction by lyso-PC of synthesis and secretion of GM-CSF by macrophages (18). The levels of GM-CSF produced by cultured macrophages in response to oxidized LDL were several orders of magnitude lower than those required to produce an increase in cell number. However, anti-GM-CSF almost completely blocked growth induction by oxidized LDL, and it was therefore suggested that GM-CSF is a “first signal” that is necessary but not sufficient for macrophage growth (18).

Oxidized LDL has also been shown to be mitogenic for bovine

* This study was supported by Grant MT8630 from the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This paper is available on line at http://www.jbc.org
and rat vascular smooth muscle cells (19). In these cells, the mitogenic effect of oxidized LDL was attributed to an oxidized phospholipid with PAF-like bioactivity and was blocked with PAF receptor antagonists, suggesting that oxidized LDL may also stimulate growth directly through PAF receptor activation. The present studies were done to clarify the mechanism by which oxidized LDL stimulates macrophage growth.

MATERIALS AND METHODS

Carrier-free Ni Cl was purchased from Mandel Scientific (Guelph, Ontario, Canada). Methyl [H]thymidine was purchased from Amersham (Cleveland, OH). RPMI 1640 medium and fetal bovine serum were obtained from Canadian Life Technologies (Burlington, Ontario, Canada). Defined fetal bovine serum was supplied by Professional Diagnostics (Edmonton, Alberta, Canada). Crotalus atrox venom phospholipase A, 4-[2-aminooctadecyl-4]-aminopropylphosphatidylcholine (C18-PC; BD-PC), 1-palmityl-2-arachidonyl PC, 1-palmitoyl-2-linoleoyl PC, and PAF were obtained from Prof. L. Birnbaum (Albany). D6-NBD-PC was a gift from Dr. John Chabala (Merck Sharp & Dohme Research, Rahway, NJ). L-659,989 was a gift from Dr. Judy Berliner (Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX). NBH3 (16). Lyso-PC was removed from oxidized LDL by incubating 200 nmol of phospholipid with 1.5 mg of LDL protein. Oxidized LDL was performed with the same volume of PBS without EDTA. Conditions for Cu oxidation of LDL were as follows: incubation of 200 μg/ml LDL in PBS with 5 μM CuSO4 at 37 °C for 2–28 h (16, 21). Further oxidation was inhibited by the addition of 200 μM EDTA and 50 μM butylated hydroxytoluene (BHT). Methylation of LDL was performed by the sequential addition of acetic anhydride (22). Seven aliquots each of 1 ml of acetic anhydride were added at 15-min intervals to 4 ml of LDL in 2 ml of ice-cold 50% saturated sodium acetate. Methylation of LDL was done by the sequential addition of a total of 40 μl of 2% formalin to 2 mg of LDL in ice-cold PBS containing 80 mM NaC- NBH3 (16). Lyso-PC was removed from oxidized LDL by incubating 200 μg/ml LDL with 10 mg/ml fatty acid-free bovine serum albumin for 18 h at 10°C in sterile PBS (pH 7.4). A control incubation with oxidized LDL was performed with the same volume of PBS without bovine serum albumin. Both oxidized LDL samples were re-isolated by ultracentrifugation for 20 h at 10°C (d = 1.210 g/cm3) (23). Phospholipase A digestion of LDL was performed by adding 5 units of phospholipase A, in 0.2 ml of 0.1% Triton X-100, to 1.5 mg of native, acetylated, or oxidized LDL in 1 ml of PBS and incubating at 37°C for 2 h (4). The reaction was then stopped by the addition of 10 mM EDTA and refrigeration. To inactivate PAF acetylhydrolase, LDL (0.4 mg/ml) in PBS or 5% PBS in RPMI 1640 medium was treated with 0.1 mM Pefabloc for 30 min at 37°C (24). This concentration of Pefabloc consistently inhibited ≥98% of PAF acetylhydrolase activity. PAF or 2-(5-oxovaleryl) PC was incorporated into Pefabloc-treated native or acetyl LDL by incubating 200 nmol of phospholipid with 1.5 mg of LDL containing 50 μM butyldihydroxyethylene at 37°C for 4 h. Lipoproteins were dialyzed against PBS containing 10 μM EDTA and sterile filtered. TLC and phosphorus assay demonstrated an association of 55 nmol PAF/mg LDL and 90 nmol 2-(5-oxovaleryl) PC/mg LDL. Oxidized 2-arachidonyl PC or 2-linoleoyl PC was obtained by exposing 50 mg of native LDL to 0.1% peroxynitrite for 3 days at 37°C. The water-soluble product was isolated by a method described previously (25). Bovine serum albumin was added to lipoprotein samples before electrophoresis to ensure reproducible migration distances. Lipoprotein bands were visualized by staining with Fat Red. Electrophoretic mobilities are expressed relative to that of native LDL. For phospholipid analysis, lipids were extracted from 100 μg of LDL protein using chloroform/methanol (2:1), and the chloroform phase was evaporated under nitrogen. Phospholipids were separated by thin-layer chromatography on Silica gel 60-HF254 plates with chloroform/methanol/water (50:35:7). Bands corresponding to PAF from PC and lyso-PC was achieved by development with chloroform/methanol/water (50:35:7). Bands corresponding to PAF from PC and lyso-PC were scraped from the plates and assayed for phosphorus content as described previously (33). Inactivation of PAF acetylhydrolase was determined by measuring the hydrolysis of C18-PC as described above.

Cell Culture—Resident peritoneal macrophages were collected from male Fischer strain by peritoneal lavage with ice-cold Ca2+-free Dulbecco's PBS. Cells were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum and adjusted to 1 x 106 cells/ml for XTT assay, 5 x 105 cells/ml for [H]thymidine incorporation assays, and 5 x 103 cells/ml for cell counting. For the XTT and thymidine incorporation assays, 0.1 ml of cell suspension was added per well to 24-well tissue culture plates. For cell number determinations, 1.0 ml of cell suspension was added per well to 24-well tissue culture plates (Falcon, Lincoln Park, NJ). Macrophages were incubated overnight at 37°C in a humidified atmosphere of 5% CO2 in air. Nonadherent cells were then removed by gentle washing with medium. For analysis of oxidized LDL-induced cell growth, macrophages were cultured in 0.1 ml of RPMI 1640 medium containing 5% fetal bovine serum with lipoproteins for 4 days and fed every 2 days with fresh medium change. In some experiments, 0.1 mM L-659,989 was included in the growth medium to inhibit PAF acetylhydrolase activity. Lipoprotein uptake and degradation experiments were performed as described previously (26). Scavenger receptor class A type II (SR- AI/II) knockout mice were obtained from Dr. T. Kodama (University of Tokyo, Tokyo, Japan). The description of the construct and the phenotypic characterization of homozogous knockout mice have been reported elsewhere (20–28). For experiments using SR-AII-deficient macrophages, the cells were obtained and cultured in the exact manner described above.

XTT Growth Assay—Macrophage growth was determined by the XTT formazan method. This assay is based on cellular reduction of XTT by mitochondrial dehydrogenase to an orange formazan product that can be measured spectrophotometrically and correlates well with the cell number (29). Briefly, 50 μl of XTT solution (1 mg/ml XTT and 25 μM N-methyladenozyporphine methyl sulfate in RPMI 1640 medium) were added to each well and incubated for 4.5 h at 37°C. Absorbance at 450 nm was then measured with a multiwell spectrophotometer. There is a linear correlation between macrophage cell number and XTT formazan formation ranging from 2 x 103 to 5 x 105 cells/well (30).

Tritiated Thymidine Incorporation Assay—Macrophage growth was also determined by the incorporation of [3H]thymidine into cellular DNA. Briefly, 10 μl of 20 μCi/ml methyl [3H]thymidine (80 Ci/mmol) were added to each well of 96-well plates for the last 24 h of each experiment. The medium was then aspirated, and cells were washed with ice-cold 10% trichloroacetic acid to precipitate DNA and remove unincorporated label. Cells were dissolved in 0.1 N NaOH and analyzed for acid-free bone material. Radioactivity was an analyzed using a liquid scintillation counter.

Cell Counting—Macrophage cell number was determined during culture using inverted phase-contrast microscopy by counting the number of cells within four random fields of view (0.40 mm2) from two separate fields.

Analytic Procedures—Protein determination was done by the method of Lowry et al. (31) in the presence of 0.05% sodium deoxycholate to minimize turbidity. Bovine serum albumin was used as the standard. Methylation of LDL increases its chromogenicity in the Lowry assay by 143% (32); hence, the assay results for methylated LDL were corrected by this factor. Lipoprotein electrophoresis was done using a Corning apparatus and Universal agarose film in 50 mM barbital buffer (pH 8.6). Bovine serum albumin was added to lipoprotein samples before electrophoresis to ensure reproducible migration distances. Lipoprotein bands were visualized by staining with Fat Red. Electrophoretic mobilities are expressed relative to that of native LDL. For phospholipid analysis, lipids were extracted from 100 μg of LDL protein using chloroform/methanol (2:1), and the chloroform phase was evaporated under nitrogen. Phospholipids were separated by thin-layer chromatography on Silica gel 60-HF254 plates with chloroform/methanol/water (50:35:7). Bands corresponding to PAF from PC and lyso-PC was achieved by development with chloroform/methanol/water (50:35:7). Bands corresponding to PAF from PC and lyso-PC were scraped from the plates and assayed for phosphorus content as described previously (33). Inactivation of PAF acetylhydrolase was determined by measuring the hydrolysis of C18-PC as described previ-
RESULTS

Oxidized LDL Induces Macrophage Cell Growth—To verify that oxidized LDL was capable of stimulating the proliferation of mouse peritoneal macrophages, cells were incubated with 30 μg/ml native or modified LDL in 5% FBS. In situ cell counts were done every 2 days for up to 10 days. As shown in Fig. 1A, oxidized LDL caused a progressive increase in cell number, comparable to that seen with 10 ng/ml GM-CSF. Neither native nor acetyl LDL increased the macrophage cell number. Cells incubated with oxidized LDL showed a substantial increase in size as well as a change to a more spindle-shaped morphology with elongated cytoplasmic extensions. Concentrations of oxidized LDL greater than 40 μg/ml appeared to be cytotoxic, as reflected by membrane blebbing, cell detachment, and a decrease in the proportion of cells that excluded trypan blue. Fig. 1B shows that the increase in cell number was accompanied by a 5-fold increase in thymidine incorporation in macrophages treated with 30 μg/ml oxidized LDL for 6 days, whereas acetyl LDL had no effect.

Macrophage Growth Is Dependent on the Degree of LDL Oxidation—For convenience, subsequent studies of the effect of native or modified LDL on the growth of murine resident peritoneal macrophages were done with the XTT reduction assay (30). The XTT assay is sensitive enough to be applied to cells grown in 96-well plates without a medium change in RPMI 1640 medium with 5% FBS containing the indicated concentrations of LDL oxidized by exposure to copper for 2 (●), 5 (○), 10 (■), 15 (▲), or 24 (▲) h. Fifty μl of XTT solution was added to each well for 4.5 h at 37 °C, and the formation of XTT formazan product was detected with a microplate spectrophotometer at 450 nm. The values shown represent the mean ± standard error of one experiment.

Macrophage Growth Stimulation Is Dependent on Internalization of the Oxidized LDL Particle—SR-AI/II is responsible for about 30% of the uptake of oxidized LDL by murine macrophages (26). Hence, if oxidized LDL uptake is essential for growth stimulation in macrophages, one would expect a proportionate decrease in growth stimulation in SR-AI/II-deficient lipids or apoB associated with very extensive oxidation are required for growth stimulation. Table I also shows that the PC content of LDL decreased progressively during oxidation, falling to one-half of the initial value after 24 h of oxidation. Sphingomyelin content remained constant under these conditions. About 70% of the lost PC was converted to lyso-PC, and the rest presumably remains as oxidized PC. Two factors contribute to the incomplete conversion of oxidized PC to lyso-PC: (a) some oxidized PC species contain long-chain polar acyl derivatives that are poor substrates for PAF acetylhydrolase (17), and (b) PAF acetylhydrolase is inactivated during LDL oxidation, and very little activity remains beyond 10 h of oxidation.
TABLE I
Phospholipid analysis and electrophoretic mobilities of modified LDLs

|                        | PC   | lypo-PC | Electrophoretic mobility |
|------------------------|------|---------|--------------------------|
| n mol PO₄/mg protein   |      |         |                          |
| Native LDL             | 677 ± 52 | 25 ± 10 | 1.00                     |
| 2-h Oxidation          | 657 ± 45 | 68 ± 27 | 1.33 ± 0.16              |
| 5-h Oxidation          | 500 ± 12 | 126 ± 20| 1.92 ± 0.08              |
| 10-h Oxidation         | 442 ± 46 | 186 ± 19| 2.61 ± 0.11              |
| 15-h Oxidation         | 375 ± 64 | 229 ± 15| 3.03 ± 0.13              |
| 24-h Oxidation         | 322 ± 45 | 247 ± 13| 3.96 ± 0.14              |
| Albumin-treated oxidized LDL | 210 ± 13 | 32 ± 3  | 3.55 ± 0.20              |
| Mock-incubated oxidized LDL | 245 ± 23 | 195 ± 29| 3.80 ± 0.21              |
| PLA₂-treated native LDL | 50 ± 6  | 527 ± 57| 1.66 ± 0.25              |
| Acetyl LDL             | 607 ± 58 | 51 ± 10 | 4.35 ± 0.10              |
| PLA₂-treated acetyl LDL | 39 ± 6  | 514 ± 49| 4.54 ± 0.13              |
| Oxidized methylated-LDL| 211 ± 15 | 206 ± 19| 3.2 ± 0.2               |

macrophages compared with controls. On the other hand, if uptake was not required or at least not rate limiting, then one would expect no difference between controls and SR-AI/II knockouts in growth stimulation by oxidized LDL. Fig. 3 shows that there was indeed less growth stimulation by oxidized LDL in SR-AI/II-deficient macrophages compared with controls. The magnitude of the decrease in growth stimulation (about one-third) is similar in magnitude to the decrease in oxidized LDL uptake by these cells. Hence, it is likely that SR-AI/II is not essential for growth stimulation but simply provides an additional pathway for the internalization of oxidized LDL. Similar results were reported previously by Sakai et al. (14), although this group concluded that SR-AI/II was necessary for growth stimulation. To determine the effect of blocking internalization via all scavenger receptors, we exploited a previous observation from our laboratory that methylation of LDL lysine residues before oxidation prevents their modification by lipid peroxidation products, and that oxidized methyl LDL is not internalized by macrophage scavenger receptors (25). As shown in Fig. 4, the total uptake of oxidized methyl-LDL was reduced by 74% compared with that of control oxidized LDL, and the effect on macrophage growth was attenuated by 84%. These results suggest that lysine modification by lipid peroxidation products (and presumably the internalization of oxidized LDL by macrophages) is necessary in order to elicit a growth stimulatory response.

The Role of Lypo-PC in Oxidized LDL-induced Macrophage Cell Growth—To test the hypothesis advanced by Sakai et al. (13) that lypo-PC accounts for the growth stimulatory effect of oxidized LDL, we tested the effect of oxidized LDL that had been depleted of lypo-PC by incubation with fatty acid-free bovine serum albumin. As shown in Fig. 5A, macrophage growth stimulation by oxidized LDL after treatment with albumin was about 80% compared with that of control oxidized LDL, even though albumin treatment had removed more than 97% of the lypo-PC (Table I). Because the incubation with albumin and the subsequent re-isolation may have caused changes other than simply removing lypo-PC, a second experiment was performed in which the formation of lypo-PC from oxidized PC during LDL oxidation was prevented by inactivating PAF acetylhydrolase with Pefabloc (24). It has been previously shown that when PAF acetylhydrolase activity is inhibited, oxidation of LDL results in the accumulation of oxidized PC compounds, some of which contain short-chain polar acyl fragments in the sn-2 position (34). When LDL was treated with Pefabloc before oxidation, the formation of lypo-PC was less than 10% of that of the control oxidized LDL, although the amount of PC consumed (oxidized) was the same. However, Pefabloc pretreatment did not reduce the ability of oxidized LDL to stimulate growth (data not shown). These results indicate that lypo-PC cannot account for more than a small part of the growth stimulatory effect of oxidized LDL. However, we could not exclude the possibility that lypo-PC might be capable of stimulating growth under some conditions. To test this, we treated native and acetyl LDL with phospholipase A₂ and then determined their effects on macrophage growth. Table I shows that treatment of native or acetyl LDL with PLA₂ resulted in the conversion of more than 90% of PC to lypo-PC. Oxidized LDL contained 60% less lypo-PC than PLA₂-treated acetyl LDL but was 7- to 10-fold more potent than PLA₂-treated acetyl LDL in inducing macrophage growth (Fig. 5B). When expressed as the amount of lypo-PC delivered to the cytosol, the difference is even greater because the rate of uptake of acetyl LDL and extensively oxidized LDL by macrophages is the same, but the degradation of oxidized LDL is much less efficient than that of acetyl LDL (35). PLA₂-treated native LDL had no effect on growth, indicating that lipoprotein internalization and not simply the transfer of lypo-PC to the plasma membrane was required for growth stimulation.

Macrophage Growth Stimulation by Oxidized LDL Is Partially Inhibited by PAF Receptor Antagonists, but PAF Itself Does Not Induce Growth—Heery et al. (19) reported that the growth of smooth muscle cells is stimulated by oxidized PC through activation of the PAF receptor. Because the results described above might be explained by oxidized phospholipids causing the growth stimulation in macrophages, the PAF receptor seemed to be a good candidate to test as a mediator of this effect. Accordingly, macrophages were treated with the PAF receptor inhibitors L-659.989 or WEB 2086 during incubation with oxidized LDL. Fig. 6A shows that L-659.989 blocked 50% of the macrophage growth stimulation by oxidized LDL. There was no morphologic evidence of cytotoxicity at
concentrations of up to 25 mM, and this drug had no effect on macrophage growth stimulation induced by 10 nM GM-CSF. However, only 20% of the macrophage growth stimulation by oxidized LDL was blocked by 20 mM WEB 2086. These inhibitor concentrations are sufficient to completely block the effect of PAF on neutrophil adhesion (19). PAF alone added to macrophages at concentrations of up to 1 mM had no effect on growth stimulation (data not shown). The lack of effect of PAF cannot be attributed to its hydrolysis to lyso-PAF, because serum PAF acetylhydrolase present in the growth medium had been inactivated by pretreatment with 0.1 mM Pefabloc, and the stability of PAF during incubation was verified by thin-layer chromatography. Because internalization of oxidized LDL is necessary to elicit a growth stimulatory response in macrophages, the growth effect of PAF that had been incorporated into Pefabloc-treated acetyl LDL was also tested. No increase in growth was observed with PAF incorporated into acetyl LDL compared with untreated acetyl LDL (Fig. 6B). These observations are compatible with the hypothesis that macrophage growth stimulation is due to oxidized phospholipids that do not act via the PAF receptor, but possibly through a novel receptor that is partly inhibited by L-659,989.

Concentrations of oxidized phosphatidylcholines account for only a small part of the growth stimulatory effect of oxidized LDL—To directly test the hypothesis that oxidized PC might be the cause of growth, we tested the effect of autoxidized 2-arachidonyl PC, 2-linoleoyl PC, or 2-(5-oxovaleroyl) PC, alone or after incorporation into native LDL or acetyl LDL. Fig. 7 shows that the autoxidized phospholipids were capable of stimulating growth to a modest extent when incorporated into native LDL or acetyl LDL, but their potency was much less than that of oxidized LDL. Acetyl LDL containing 2-(5-oxovaleroyl) PC or 2-(5-oxovaleroyl) PC alone did not stimulate growth (data not shown). To determine whether oxidized PCs accounted for a significant part the growth-stimulating effect of oxidized LDL, the latter was incubated with PLA2 under conditions that caused a near complete hydrolysis of intact and oxidized PC and then compared with untreated oxidized LDL. Surprisingly, PLA2 diges-
concentrations of oxidized LDL (by TLC at the end of the incubation verified that PAF had not been degraded. Residual PAF acetylhydrolase activity in Pefabloc-treated acetyl LDL was less than 1%. Analysis of medium containing PAF-enriched acetyl LDL was carried out with macrophages incubated with 10 ng/ml GM-CSF (●). Cell growth was measured using the XTT assay. Each value represents the mean ± standard error of triplicate determinations from two experiments. *, p ≤ 0.005.

Partial inhibition of oxidized LDL-induced macrophage growth by PAF receptor antagonists. A, macrophages were preincubated for 30 min with varying concentrations of the PAF receptor antagonists L-659,989 (●) or WEB 2086 (■), and then 30 μg/ml oxidized LDL was added for 4 days in the continued presence of each antagonist. As a control for nonspecific toxicity, parallel incubations of L-659,989 were carried out with macrophages incubated with 10 ng/ml GM-CSF (○). Cell growth was measured by the XTT assay. Each value represents the mean ± standard error of triplicate determinations from two experiments. *, p ≤ 0.005. B, macrophages were incubated with the indicated concentrations of oxidized LDL (●), Pefabloc-treated acetyl LDL (□), or Pefabloc-treated acetyl LDL containing 55 nmol PAF/mg LDL protein (■). Residual PAF acetylhydrolase activity in Pefabloc-treated acetyl LDL was less than 1%. Analysis of medium containing PAF-enriched acetyl LDL by TLC at the end of the incubation verified that PAF had not been degraded.

**FIG. 6.** Partial inhibition of oxidized LDL-induced macrophage growth by PAF receptor antagonists. A, macrophages were preincubated for 30 min with varying concentrations of the PAF receptor antagonists L-659,989 (●) or WEB 2086 (■), and then 30 μg/ml oxidized LDL was added for 4 days in the continued presence of each antagonist. As a control for nonspecific toxicity, parallel incubations of L-659,989 were carried out with macrophages incubated with 10 ng/ml GM-CSF (○). Cell growth was measured by the XTT assay. Each value represents the mean ± standard error of triplicate determinations from two experiments. *, p ≤ 0.005. B, macrophages were incubated with the indicated concentrations of oxidized LDL (●), Pefabloc-treated acetyl LDL (□), or Pefabloc-treated acetyl LDL containing 55 nmol PAF/mg LDL protein (■). Residual PAF acetylhydrolase activity in Pefabloc-treated acetyl LDL was less than 1%. Analysis of medium containing PAF-enriched acetyl LDL by TLC at the end of the incubation verified that PAF had not been degraded.

**FIG. 7.** Effect of oxidized PC on growth. Autoxidized 2-linoleoyl PC or 2-arachidonyl PC were incorporated into native LDL or acetyl LDL as described under “Materials and Methods.” The indicated concentration of modified LDL was then incubated with macrophages for 4 days, and macrophage growth was measured using the XTT assay. Oxidized LDL, ○; acetyl LDL modified with oxidized 2-arachidonoyl PC, ●; native LDL modified with 2-arachidonyl PC, ■; acetyl LDL modified with oxidized 2-linoleoyl PC, □; native LDL modified with oxidized 2-linoleoyl PC, ▲; acetyl LDL, ●. The range of values for incorporation of oxidized PC into LDL was 420–558 nmol/mg LDL protein. Data in Fig. 8: A show that LDL modified with oxidation products derived from linoleic acid or arachidonic acid was comparable in potency to oxidized LDL in stimulating growth. To evaluate the extent of modification by fatty acid peroxidation products necessary to stimulate growth, 1 mg of LDL was treated with oxidation products from 0.2, 0.4, 0.8, or 1.2 mg of arachidonic acid. The resulting LDLs had electrophoretic mobilities that were 1.3-, 2.7-, 3.6-, and 4.4-fold that of native LDL, respectively. As judged by these changes in electrophoretic mobility (25), we estimate that derivatization of at least 15% of lysine residues was required for detectable growth induction. Surprisingly, bovine albumin and human high density lipoprotein modified by arachidonic acid oxidation products also stimulated growth, although the effect on growth was linear with concentration as opposed to saturable, as with modified LDL (Fig. 8B). Nevertheless, the results in Fig. 8 are compelling evidence that an oxidative modification of apoB rather than oxidized lipid(s) per se accounts for most of the growth induction by oxidized LDL.

**DISCUSSION**

Sakai et al. (13) were the first to describe the stimulation of macrophage growth by oxidized LDL. This group concluded that the effect was due to lyso-PC and required lipoprotein internalization by SR-AI/II (13, 14). Although these interpretations conflict with ours, the actual data are in general agreement. Specifically, we confirmed their findings that PLA₂-treated acetyl LDL can mimic the effect of oxidized LDL and that macrophages lacking scavenger receptors are less responsive to oxidized LDL than control cells. However, we found that the removal of more than 97% of lyso-PC from oxidized LDL did not substantially reduce its effect on growth stimulation and that oxidized LDL was almost an order of magnitude more potent than PLA₂-treated acetyl LDL, which contained twice as much lyso-PC per particle as oxidized LDL. Of note, a recent study from the Japanese group also reported that oxidized LDL was more potent than PLA₂-treated acetyl LDL at stimulating...
There is no oxidation of LDL lipids. Macrophages were incubated for 4 days with the indicated concentrations of oxidized LDL (○), LDL modified with oxidation products derived from 1.2 μg of linoleic acid (●), or LDL modified with oxidation products derived from 0.2 (□), 0.4 (●), 0.8 (■), or 1.2 mg of arachidonic acid (▲), and then cell growth was measured with the XTT assay.

**Fig. 8. Stimulation of macrophage growth by oxidation product-modified LDL.**

A, LDL was modified with fatty acid peroxidation products as described under “Materials and Methods.” This procedure results in derivatization of LDL lysine residues with reactive aldehydes, but there is no oxidation of LDL lipids. Macrophages were incubated for 4 days with the indicated concentrations of oxidized LDL (○), LDL modified with oxidation products derived from 1.2 μg of linoleic acid (●), or LDL modified with oxidation products derived from 0.2 (□), 0.4 (●), 0.8 (■), or 1.2 mg of arachidonic acid (▲), and then cell growth was measured with the XTT assay. B, bovine serum albumin and human high density lipoprotein were modified as described above with arachidonic acid oxidation products. Macrophages were incubated as described above with the indicated concentrations of native albumin (○), albumin modified with oxidation products (●), high density lipoprotein (■), or high density lipoprotein modified by oxidation products (▲), and then cell growth was measured. Similar results were obtained in two replicate experiments.

Macrophage growth (18). Hence, the data from both laboratories indicate that the effect of oxidized LDL on growth cannot be attributed entirely to lyso-PC. The difference in findings with SR-AI/II-deficient mice appear to be minor: Sakai et al. (14) reported that about 50–70% of oxidized LDL uptake and a similar proportion of the growth induction were mediated by SR-AI/II, whereas we found that only about 30% of oxidized LDL uptake and 30% of growth induction were mediated by this receptor. This discrepancy could be explained by differences in oxidized LDL preparations between laboratories or by the different genetic backgrounds of the SR-AI/II-deficient transgenic mice that were used.

Heery et al. (19) analyzed the effects of oxidized LDL on the growth of arterial smooth muscle cells. They found that oxidized LDL (but not native LDL) induced smooth muscle cell growth, and that this effect was mimicked by PAF and could be blocked by the PAF receptor antagonists WEB 2086 and L-659,989. Furthermore, it was shown that growth stimulation was associated with a lipid fraction intermediate in polarity between PC and PAF, consistent with oxidized PC. The biological activity of this component was eliminated by PAF acetylhydrolase, and, in fact, active fractions could only be recovered from LDLs that had been pretreated with diisopropylfluorophosphate to inactive PAF acetylhydrolase before oxidation. These observations in smooth muscle cells differ substantially from our findings in macrophages in that PAF itself did not induce growth in macrophages, and WEB 2086 had only a minimal inhibitory effect on growth. Oxidized PC itself was found to cause some growth induction in macrophages, but because the growth effect of oxidized LDL could not be abolished by PLA₂, and the growth was mimicked by LDL modified with fatty acid oxidation products (and containing no oxidized PC), we concluded that protein modification was predominantly responsible for growth induction.

To date, there has been no detailed characterization of the structure of the oxidized phospholipids in oxidized LDL that are responsible for PAF receptor activation. Stremler et al. (36, 37) identified 1-palmitoyl-2-[5-oxovaleroyl] PC in oxidized LDL and showed that this compound is a substrate for PAF acetylhydrolase and can increase thymidine incorporation in smooth muscle cells (19). However, the maximal effect of 2-[5-oxovaleroyl] PC was only a 33% increase over control, whereas PAF and oxidized LDL more than doubled thymidine incorporation. This suggests that there may be compounds more potent than 2-[5-oxovaleroyl] PC that account for most of the growth stimulation. Indeed, in our studies in macrophages, 2-[5-oxovaleroyl] PC had no effect on growth. Our finding that the active components could not be removed by preincubation of oxidized LDL with albumin suggests that the active fraction is not a highly polar PC derivative, but part of the growth-promoting activity could be due to a condensation product of oxidized PC or a form with a residue longer than 5-oxovaleroyl at the 2 position. One such PC oxidation product could be F₂-isoprostanoyl PC, which has been shown to be formed during nonenzymatic oxidation of LDL (38). Alternatively, it could be a derivative of 2-linoleoyl PC that cannot generate a derivative containing a short-chain aldehyde.

Reconciliation of the findings in smooth muscle cells with those in macrophages requires that there are two pathways for growth induction: one active in smooth muscle cells that involves the PAF receptor and PAF-like forms of oxidized phosphatidylcholine, and a second pathway that involves a modification of apoB components present only in very extensively oxidized LDL and mediated by a different and possibly novel receptor. Additional studies are required to define the domain of oxidized apoB that is involved in growth stimulation and to determine whether this is mediated by a cell surface receptor or is a consequence of the internalization and processing of modified apoB. Once the stimulatory component and its receptor have been identified, it should be possible to clarify the signal transduction pathways involved in macrophage growth stimulation. Evidence has been presented to implicate phosphatidylinositol 3-kinase, protein kinase C, and protein tyrosine kinases (15, 30), but there are discrepancies between laboratories in the relative importance of these pathways in growth induction by oxidized LDL.

Although there is strong evidence that oxidatively modified LDL is present in atherosclerotic lesions (39–41), it has not been proven that LDL in early or developing lesions in man is oxidized to the extent that we found to be necessary for macrophage growth induction in vitro (42). However, even mildly oxidized LDL is capable of inducing colony-stimulating factor
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gene expression in endothelial cells (43), and macrophage colony-stimulating factor together with other endothelial-derived factors are synergistic stimuli for macrophage growth (44). Furthermore, induction of macrophage colony-stimulating factor has been demonstrated in foam cell macrophages in human and rabbit atherosclerotic lesions (45). Hence, it is possible that a lesser degree of oxidation, comparable to what is present in early lesions in vivo, might have a stimulatory effect on macrophage growth in vivo.

The ultimate importance of in situ proliferation of macrophages in the artery wall during the development of atherosclerotic lesions is unknown. However, the present findings provide further insights into a possible mechanistic explanation for the macrophage proliferation in atherosclerotic lesions demonstrated by immunocytochemical studies (10, 11) and suggest an additional role for oxidized LDL in the pathogenesis of atherosclerosis.

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