Oxidized Low Density Lipoprotein Inhibits the Migration of Aortic Endothelial Cells In Vitro

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Abstract. Endothelial cell (EC) migration is a critical and initiating event in the formation of new blood vessels and in the repair of injured vessels. Compelling evidence suggests that oxidized low density lipoprotein (LDL) is present in atherosclerotic lesions, but its role in lesion formation has not been defined. We have examined the role of oxidized LDL in regulating the wound-healing response of vascular EC in vitro. Confluent cultures of bovine aortic EC were "wounded" with a razor, and migration was measured after 18 to 24 h as the number of cells moving into the wounded area and the mean distance of cells from the wound edge. Oxidized LDL markedly reduced migration in a concentration- and oxidation-dependent manner. Native LDL or oxidized LDL with a thiobarbituric acid (TBA) reactivity <5 nmol malondialdehyde equivalents/mg cholesterol was not inhibitory; however, oxidized LDL with a TBA reactivity of 8–12 inhibited migration by 75–100%. Inhibition was half-maximal at 250–300 μg cholesterol/ml and nearly complete at 350–400 μg/ml. The antimigratory activity was not due to cell death since it was completely reversed 16 h after removal of the lipoprotein. The inhibitor molecule was shown to be a lipid; organic solvent extracts of oxidized LDL inhibited migration to nearly the same extent as the intact particle. When LDL was variably oxidized by dialysis against $FeSO_4$ or $CuSO_4$, or by UV irradiation, the inhibitory activity correlated with TBA reactivity and total lipid peroxides, but not with electrophoretic mobility or fluorescence (360 ex/430 em). This indicates that a lipid hydroperoxide may be the active species. These results suggest the possibility that oxidized LDL may limit the healing response of the endothelium after injury.

ENDOTHELIAL cell (EC)[1] migration and proliferation are critical processes in the formation of blood vessels and in the repair of injured vessels. In the earliest report that distinguished between the roles of migration and proliferation in vivo, Schoefl (52) observed that EC migration was the initiating, and potentially rate-limiting event in the regeneration of capillaries after tissue injury. Ausprunk and Folkman (2) likewise observed that migrating EC initiated the extension of capillary "buds" towards angiogenic factors. Both laboratories concluded that migrating cells at the tip of a new capillary started the process and that subsequent EC proliferation filled in the gaps left behind by vacated cells. The same EC processes are involved in the repair of major blood vessels after the physical trauma induced by balloon angioplasty, vascular reconstruction or replacement, and organ implantation. Haudenschild and Schwartz (23) showed that removal of the endothelium from a rat thoracic aorta with a balloon catheter was followed by a gradual regeneration of the endothelial lining. In this system, too, the repair process was initiated by EC migration and followed by proliferation. Reidy and Schwartz (46) subsequently observed that a very narrow wound to the endothelium of the rat aorta, ~1 to 2 cells in width, could be completely healed within 8 h without any evidence of cell replication.

The apparent role of EC migration in vessel neogenesis and repair in vivo motivated studies on the regulation of this process in vitro. Sholley et al. (55) reported that mechanically wounded human umbilical vein EC initiated an in vitro wound-healing process closely resembling that observed in vivo. Migration was seen within 12 h of the injury and significant wound repair within 24 h, well before any cell proliferation which began after 36 h. In addition, exposure of cultured EC to 1,500 rad of x-rays completely abolished cell proliferation but did not alter the rate of repair by migration.

A search for the signaling factors was initiated by Zetter (64) who showed that tumor-derived factors stimulated the migration of bovine capillary EC. Since then a number of agents have been reported to stimulate migration of EC derived from capillaries or large vessels, or both. Among these stimulatory factors are heparin (4), tumor-promoting phorbol esters (39), vascular permeability factor (12), and acidic (57) and basic (11, 50) FGF. Recent evidence suggests that basic FGF may be a critical autocrine or paracrine regulator of EC migration since cultured EC themselves synthesize and release it (primarily into the underlying matrix), and

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1. *Abbreviations used in this paper*: EC, endothelial cell; LDL, low density lipoprotein; MDA, malondialdehyde; TBARS, thiobarbituric acid-reacting substances.

since migration is substantially blocked by anti-basic FGF antibody (59). FGF may be an important physiological regulator of EC migration (and proliferation); both acidic and basic forms stimulate endothelial regeneration of vessels injured by balloon catheter in vitro (6, 33). Among the inhibitors of EC migration that have been identified, TGF-β1 and fibronectin are particularly potent, and evidence suggests that they may influence repair in vivo (35, 51).

The role of lipids and lipoproteins in cell migration has not been extensively investigated. Bürk et al. (9) showed that serum from pigs on an atherogenic diet stimulated migration of 3T3 cells to a lesser extent than control porcine serum. Kanayasu et al. (29) showed that eicosapentaenoic acid, but not arachidonic acid or docosahexaenoic acid, potentiated the migration of EC in response to serum and basic FGF. Recent observations from several laboratories suggest that oxidized lipids and lipoproteins modulate a variety of EC functions including reduction of pinocytic activity (7) and production of PDGF (21). Oxidized low density lipoprotein (LDL) also enhances monocyte adhesion to EC (5) and stimulates production of prostacyclin (58), colony-stimulating factors (44), and tissue factor (16, 61). Furthermore, oxidized LDL alters chemotactic and migratory responses in other vascular cells. It is chemotactic for smooth muscle cells (3) and monocytes (13), but inhibits migration of tissue macrophages (43). Finally, recent immunohistochemical data from several laboratories suggest that oxidized LDL is present in atherosclerotic lesions (for review see reference 62), and may in fact be present in plasma (1). These studies motivated us to examine the role of oxidized LDL on the migratory response of aortic EC.

Materials and Methods

Cell Culture and Media

EC were isolated from adult bovine aortas essentially as described (15). They were subcultured by trypsinization and grown from a 1:3 split ratio to confluence in 175 cm² flasks in DME and Ham's F12 medium (GIBCO BRL, Gaithersburg, MD) containing 5% FCS (Hyclone Labs., Logan, UT). The identity of the cells was confirmed by their nonoverlapping, cobblestone morphology, and by anti-factor VIII antigen immunofluorescence. EC were used between passages 11 and 20. All EC incubations were at 37°C in a humidified atmosphere of air containing 5% CO_2. For migration studies, the cells were grown to confluence in 1 ml of serum-containing medium in 12-well tissue culture clusters (Costar Corp., Cambridge, MA). The cells were made quiescent by changing the medium to 1 ml of serum-free DME (Sigma Chem. Co., St. Louis, MO) containing 1 mg/ml gelatin (Difco Laboratories, Detroit, MI) for at least 24 h before use. Basic FGF (human recombinant) was from Upstate Biotechnology Inc. (Lake Placid, NY). Cellular protein synthesis was measured by incorporation of [³H]leucine (New England Nuclear, Wilmington, DE) into TCA-precipitable material as described (20).

Measurement of EC Migration

EC migration was measured by the razor wound method essentially as described by Bürk (8), with modifications according to Sato and Rifkin (50). Confluent, quiescent EC cultures were wounded with a razor pressed gently through the cell sheet into the plastic well to mark the origin, and then drawn through the monolayer to remove cells on one side. The medium was aspirated and replaced with serum-free medium containing 1 mg/ml gelatin plus lipoproteins or other test materials in a total volume of 0.5 ml. Cell migration was permitted for up to 24 h and then terminated by fixing and staining with Wright-Giemsa stain (modified, Sigma Chem. Co.). Migration was quantitated by a computer-assisted procedure done by a person blinded with respect to the identity of the experimental treatments. The cul-

tures were imaged by a charge-coupled device digital camera (Sierra Scientific, Sunnyvale, CA) with a 4× objective lens on an inverted stage phase-contrast microscope (model CK-2; Olympus Corp. Precision Instr. Div., Lake Success, NY). A 256 gray-level, 640 × 480 pixel image was transferred to a Macintosh computer via a frame grabber board (Data Translation, Inc., Marlboro, MA). Image and data analysis were done using the "Image" software package provided by Dr. Wayne Rasband, National Institutes of Health. Cell nuclei were identified by a "particle analysis" algorithm using preset limits of size and density, and the perpendicular distance from the center of each nucleus to the origin line was determined and designated as the migration distance. The number of cells that crossed the origin line and the average migration distance were calculated. In each well, two randomly chosen fields, each consisting of a 1,500-μm length of wound were analyzed and the results summed. The data from duplicate wells were expressed as the mean ± standard error. All experiments were done two or more times, and representative results are shown.

Preparation of Lipoproteins

Lipoproteins were prepared from freshly drawn, citrated normolipemic human plasma to which EDTA was added before ultracentrifugation. LDL (density = 1.019–1.063) was isolated by sequential ultracentrifugation as described previously (25). The purity was assessed by gel electrophoresis, and all preparations were assayed for protein (34), total cholesterol (Boehringer Mannheim Corp., Indianapolis, IN), endotoxin (Pierce Chemical Co., Rockford, IL), and thiobarbituric acid (TBA) reactivity (53).

Unless otherwise indicated, LDL was oxidized by ferrous ion-catalyzed oxidation (21). In brief, EDTA was removed from aliquots of lipoprotein (containing 10–15 mg of cholesterol) by dialysis against 0.9% NaCl, pH 7.4, at 4°C for 12–18 h. The lipoprotein was then dialyzed for 6–8 h at 37°C against the same solution containing freshly prepared 5 μM FeSO₄, with dialysate changes every 2 h. The oxidation was stopped by the addition of EDTA to a final concentration of 100 μM, and the lipoprotein sterilized by passing through a 0.22-μm filter (Millipore Corp., Bedford, MA). In some preparations a modified procedure was used. Contaminating lipoprotein-bound metals were removed by extended dialysis at 4°C against saline solution containing 100 μM EDTA. Both methods gave similar results with respect to their effects on EC migration. LDL was also oxidized by dialysis against CuSO₄, and by exposure to ultraviolet irradiation for 3 h in a UV Stratalinker 1800 (Strategene Inc., La Jolla, CA) at an instrument setting of 150 (μJ × 100). Total cholesterol and TBA reactivity were determined for all preparations. Selected lipoprotein preparations were further characterized by electrophoretic mobility on an agarose gel (Corning Inc., Corning, NY), by lipid peroxide content (17), and by fluorescence spectroscopy with excitation and emission set to 360 nm and 430 nm, respectively (30). Lipid extracts from native and oxidized LDL were prepared by dialysis against saline to remove EDTA, lyophilization, acetone extraction, and reconstitution in a small volume of acetone/ethanol (1:1, vol/vol). The final concentration of acetone/ethanol in the culture medium in all experiments was <0.5% by volume, and lipid-free controls containing this amount of solvent were tested and did not alter EC migration. The amount of lipoprotein lipid was expressed per amount of cholesterol in the original sample. All solvents were from Fisher Scientific (Fair Lawn, NJ) and other reagents were from Sigma Chem. Co.

Results

A time course of migration of bovine aortic EC was performed to choose experimental conditions such that the number of migrating cells and the average distance of migration was easily measured and reproducible. The results in Fig. 1 show that cell migration was clearly visible by 8 h and increased up to at least 48 h. The number of migrating cells and average migration distance are shown in Fig. 2. Both variables were nearly linear for up to 48 h. Cells undergoing cell division were only rarely observed in any wells up to 24 h. In subsequent experiments an incubation time between 18 and 24 h was chosen to maximize statistical power while minimizing cell proliferation as a confounding variable.

The effect of native and oxidized LDL on EC migration was studied. LDL was oxidized by dialysis against FeSO₄ to a final oxidation level, measured as TBA-reacting sub-
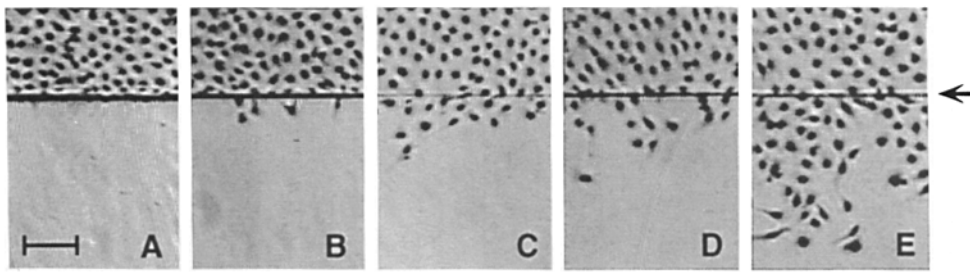
Figure 1. Time course of migration of bovine aortic EC after wounding—micrographs. Confluent cultures of bovine aortic EC were "wounded" and the cells were fixed and stained after 0 (*A*), 8 (*B*), 16 (*C*), 24 (*D*), and 48 h (*E*). The images were captured by a CCD camera and digitally enhanced to improve contrast. Bar, 100 μm.

stances, of 10.9 nmol malondialdehyde (MDA) equivalents/ mg cholesterol. The phase-contrast photomicrographs of Fig. 3 show that oxidized LDL, but not native LDL, markedly inhibited EC migration in a concentration-dependent manner. Quantitation of these results as the number of migrating cells yields a half-maximal inhibitory concentration of ~300 μg/ml, and near complete inhibition at 400 μg cholesterol/ml (Fig. 4 *A*). When migration was expressed as mean distance, a virtually identical half-maximal inhibitory concentration was found, and the migration in the presence of the highest amount of oxidized LDL was 27% of the lipoprotein-free control (not shown). These concentration response data are representative of many repetitions of this experiment using oxidized LDL between 6 and 12 nmol MDA equivalents/mg cholesterol. Similar observations were made with oxidized LDL prepared from LDL from multiple donors. In addition, the migration of multiple isolates of bovine aortic EC was inhibited by oxidized LDL. The inhibitory activity of the lipoprotein required oxidative modification since native LDL was not inhibitory at any concentration, and, in fact, LDL stimulated migration by nearly 40% compared with lipoprotein-free controls (Fig. 4 *A*).

The antimigratory activity of oxidized LDL did not appear to be due to cell death, since cells that were completely static appeared normal by phase-contrast microscopy (although at 500 μg cholesterol/ml some toxicity was observed visually). To quantitate the viability of the cells under these experimen-

tal conditions, protein synthesis was measured by incorporation of [³H]leucine into cellular protein (Fig. 4 *B*). The decrease in protein synthesis was ~10% at a concentration of oxidized LDL that half-maximally inhibited migration, and ~30% at a concentration that completely stopped migration. This decrease may be due to death of some cells, but it may also reflect a general decrease in the metabolic activity of the cells.

The inhibition of migration by lipoproteins was further characterized with respect to the extent of oxidation. LDL was variably oxidized by incubation with FeSO₄ for different lengths of time up to 8 h. Both the native and mildly oxidized LDL stimulated EC migration compared with lipoprotein-free controls (Fig. 5). This result, while not dramatic, was seen in all repetitions of this experiment. However, at higher oxidation levels, a marked inhibitory effect was observed, with half-maximal inhibition at ~5-6 nmol MDA/mg cholesterol (at 500 μg cholesterol/ml), and near-total inhibition observed at 7-8 nmol MDA/mg cholesterol.

Since oxidized LDL is toxic to many types of cultured cells including EC (24), experiments were done to rule out several toxicity-related scenarios that could explain our observations. It should be mentioned that, at least in fibroblasts, the toxic effect is primarily limited to proliferating cells during S-phase of the cell cycle (31). If this result can be extended to EC, this mechanism is unlikely to apply to our observations of unstimulated cells in serum-free medium. We nonetheless considered the possibility that the oxidized LDL-treated cells were lethally injured, but that the injury was not apparent morphologically. To test this, the reversibility of the treatment with oxidized LDL was examined. Confluent EC cultures were wounded and then incubated for 24 h with oxidized LDL at a concentration that inhibited migration by ~80% (Fig. 6 *A*). After replacement of the medium with lipoprotein-free medium, a near-normal level of cell migration was observed during the subsequent 2-d recovery period. In other experiments, similar reversibility was observed after incubation of EC with oxidized LDL for up to 96 h (not shown). To more accurately determine the recovery time, another experiment was done using shorter incubation periods to provide information on the initial rate of migration. EC were preincubated with oxidized LDL for 12 h (a time that was sufficient to almost completely inhibit migration), and then the medium was replaced with lipoprotein-free medium. After several wash-out periods up to 24 h, the culture was wounded and cell migration measured during an 8-h interval. Immediately after removal of the oxidized LDL (indicated as 0 h), the number of migrating
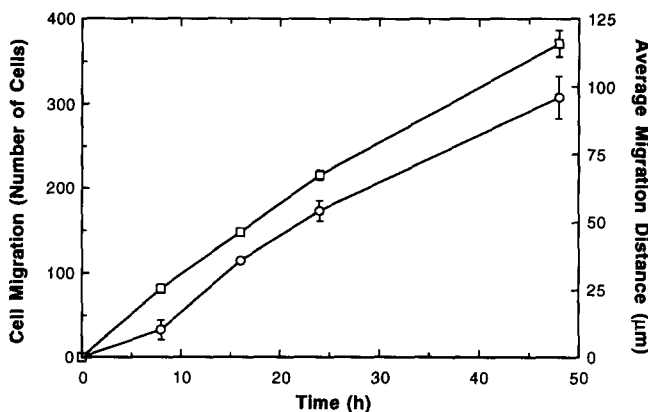

Figure 2. Time course of migration of bovine aortic EC after wounding—quantitation. The number of cells that have traversed two 1,500-μm lengths of the original wound edge (□) and the average net migration distance (○) are shown (mean and standard error of duplicate wells).
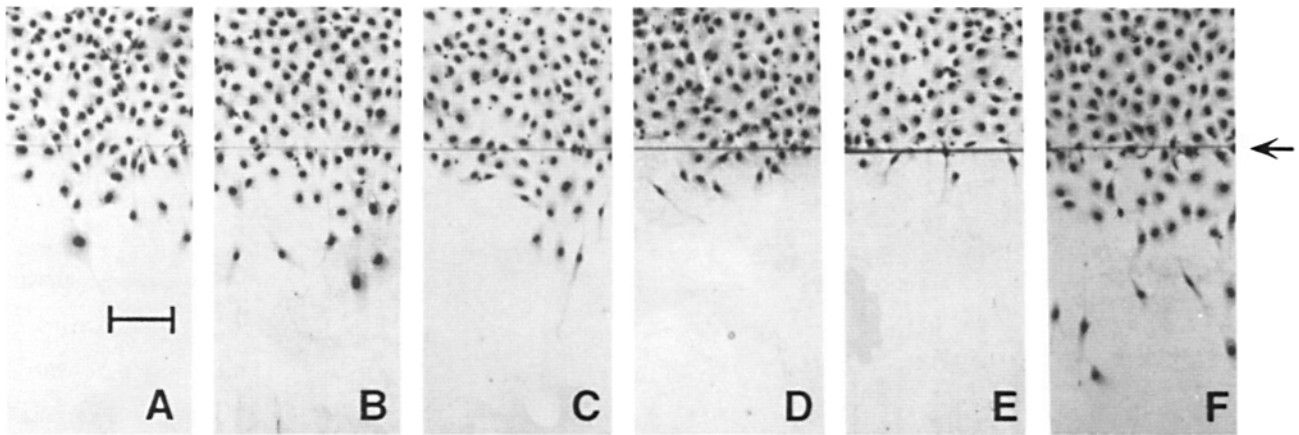
Figure 3. Effect of native and oxidized LDL on EC migration—photomicrographs. Native LDL (TBARS = 1.2 nmol MDA equivalents/mg cholesterol) was oxidized by dialysis against 5 μM FeSO$_4$ at 37°C for 8 h (TBARS = 10.9 nmol MDA equivalents/mg cholesterol). EC migration was stopped after incubation with lipoproteins for 20 h, and the cells were fixed and stained with modified Wright-Giemsa stain. (*A*) Lipoprotein-free control; (*B*) oxidized LDL, 100 μg cholesterol/ml; (*C*) oxidized LDL, 250 μg cholesterol/ml; (*D*) oxidized LDL, 300 μg cholesterol/ml; (*E*) oxidized LDL, 350 μg cholesterol/ml; and (*F*) native LDL, 400 μg cholesterol/ml. Bar, 100 μm.
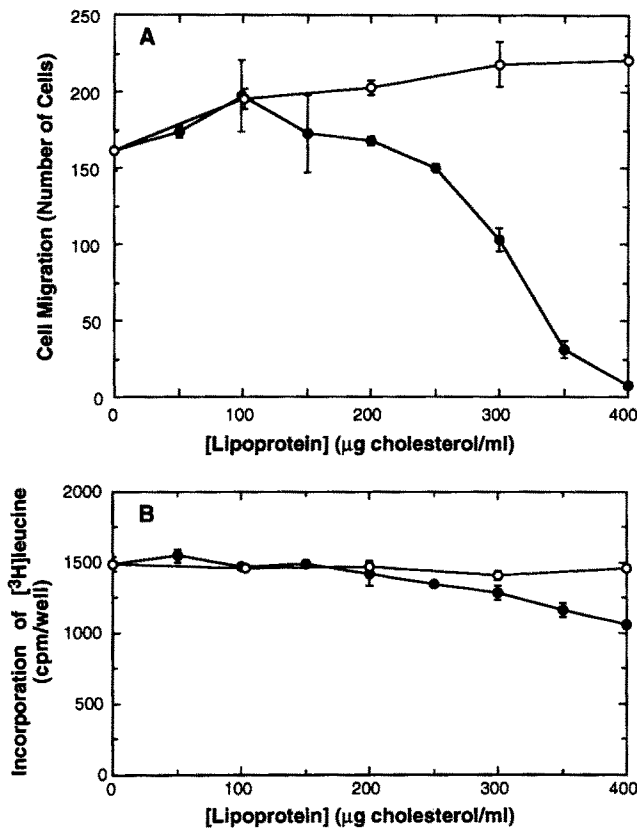

Figure 4. Effect of native and oxidized LDL on EC migration—quantitation. (*A*) EC were incubated with native (o) and oxidized LDL (●) as described in Fig. 3. The number of migrating cells in duplicate wells (mean ± SEM) was quantitated by computer-assisted particle analysis. Similar trends were seen for the effect of lipoproteins on the mean migration distance. (*B*) [³H]Leucine (1 μCi/ml) was added to parallel wells, in duplicate, during the last 2 h of the 20-h incubation, and cellular protein synthesis measured as TCA-precipitable protein (mean and standard error are shown).

cells increased from almost none to ~25% of the untreated control (Fig. 6 *B*). The cell migration rate increased to half of the control rate after 8 h and completely returned to the control rate after 16 h. These data confirm those of Fig. 6 *A* that show that the antimigratory activity of oxidized LDL is completely reversible.

In an alternative scenario, oxidized LDL could be lethal only to migrating cells, and not to the confluent cells remaining behind the cut edge. In this case, cells that migrated away from the cell sheet would be killed by the oxidized LDL, and since dead cells lift away from the dish, they would not appear as migrating cells in our assay. This possibility was tested by time-lapse videomicrography of EC (at 10-min in-
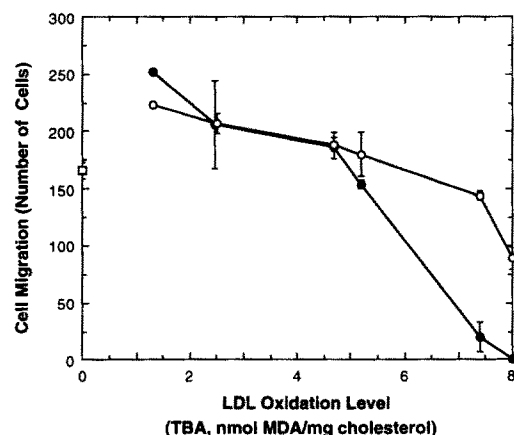

Figure 5. Effect of oxidation level on EC migration. LDL was oxidized by dialysis against 5 μM FeSO$_4$ at 37°C for various times up to 8 h. EC migration was measured after incubation without lipoprotein (□), with lipoprotein added at 300 μg cholesterol/ml (o), or with lipoprotein at 500 μg cholesterol/ml (●) for 24 h. Mean and standard error are shown.
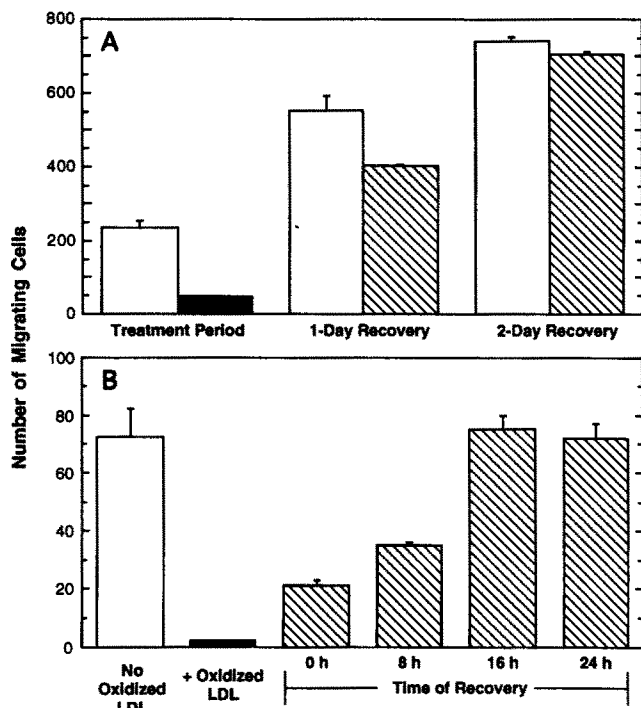
Figure 6. Recovery of EC migration after removal of oxidized LDL. (*A*) Confluent cultures of EC were incubated for 1 d in the presence or absence of oxidized LDL (400 μg cholesterol/ml, TBARS = 7.3 nmol MDA/mg cholesterol), the medium was replaced with lipoprotein-free medium, and cell migration was allowed to continue during the second and third day. After each day, duplicate EC cultures were fixed and stained, and cell migration was determined. The number of migrating cells in cultures of untreated EC (*open bars*), EC treated with oxidized LDL (*black bar*), and EC treated with oxidized LDL followed by lipoprotein-free medium (*striped bars*) are shown as the mean and standard error. (*B*) Confluent EC were treated for 12 h in the presence of oxidized LDL (300 μg cholesterol/ml, TBARS = 13.0 nmol MDA/mg cholesterol). The medium was replaced with lipoprotein-free medium for up to 24 h, the cultures were wounded, and migration was measured after an 8-h period. One control pair of wells was not pretreated with oxidized LDL (*open bar*), in a second pair the oxidized LDL was replaced with fresh oxidized LDL after the wound was made (*black bar*). In the remaining wells, the oxidized LDL was replaced with medium, and the cells were allowed to recover for 0, 8, 16, and 24 h before wounding (*striped bars*). The number of migrating cells are indicated as the mean and standard error.

tervals) in the presence of oxidized LDL. At a concentration of oxidized LDL that totally blocked migration, all cells that were in the view field at the beginning of the experiment were accounted for after 24 h, establishing that cell death was not responsible for the inhibition of migration (data not shown). After replacement of the medium with lipoprotein-free medium, EC migration resumed after ~8 h, further confirming both the lack of toxicity and the time course of Fig. 6 *B*.

Basic FGF is a potent stimulator of EC migration and studies with neutralizing antibodies have shown that endogenous basic FGF is required for migration (59). We have previously shown that oxidized LDL reduces the production and release from bovine aortic EC of a distinct factor with mitogenic and angiogenic properties, i.e., PDGF (21). As an ini-

Table I. Effect of Exogenous Basic FGF on the Antimigratory Activity of Oxidized LDL

	Mean migration distance	Control
	μM	*%*
Control (no addition)	58 ± 2	—
+ basic FGF	106 ± 2	183
+ oxidized LDL	36 ± 2	62
+ basic FGF, oxidized LDL	32 ± 3	55

Confluent bovine aortic EC were wounded and incubated with oxidized LDL (TBARS = 10.9 nmol MDA/mg cholesterol, 400 μg cholesterol/ml), basic FGF (10 ng/ml), or both. After 24 h, the cells were fixed and stained, and cell migration in duplicate wells was measured (mean ± standard error).

tial approach to the mechanism of its antimigratory activity, we explored the possibility that the inhibition by oxidized LDL was due to decreased release of basic FGF. In this case, exogenously supplied growth factor would be expected to restore normal migration rates to EC treated with oxidized LDL. Table I shows that the inhibitory activity of the lipoprotein was not affected by basic FGF at a concentration that maximally stimulates migration, thus indicating an alternative mechanism of action.

To determine the chemical nature of the inhibitor molecule, the effect of lipid extracts of the lipoprotein preparations on EC migration was examined. Lipids from native and oxidized LDL were prepared by lyophilization, extraction by acetone, and reconstitution with acetone/ethanol. The oxidized lipid extract significantly inhibited migration; however, the potency of the extract was approximately half that of the intact particles (Fig. 7). The extract from native LDL
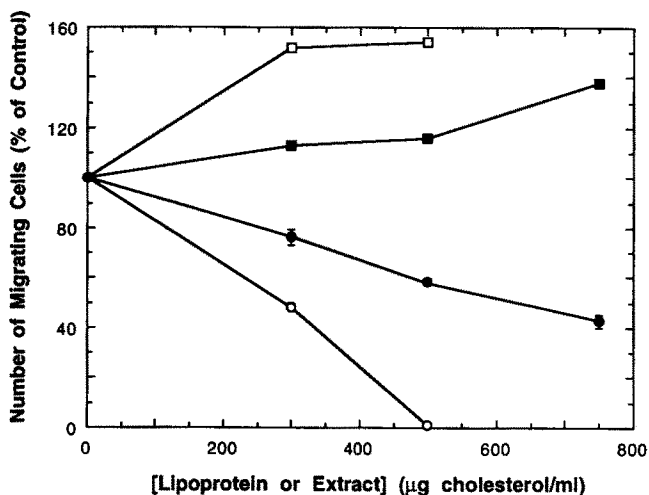

Figure 7. The activity of lipid extracts of native and oxidized LDL. LDL was oxidized by incubation with 5 μM $FeSO_4$ for 8 h (TBARS = 7.8 nmol MDA/mg cholesterol). The lipids from native and oxidized LDL were extracted by lyophilization, followed by acetone extraction, and reconstitution with acetone/ethanol (1:1, vol/vol, TBARS = 5.1 nmol MDA/mg cholesterol). The extracts were added to media and briefly sonicated before addition to cells. Cell migration (mean ± standard error) was measured after 20 h in the presence of the LDL (□) and its lipid extract (■), and oxidized LDL (○) and its lipid extract (●).

Table II. Degree of Oxidation of LDL Modified by Different Methods

LDL treatment	Peroxides (nmol LPO/mg cholesterol)	TBARS (nmol MDA/mg cholesterol)	Relative fluorescence (arbitrary units)	Relative electrophoretic mobility
None	21	0.2	21	1.0
Fe²⁺	374	6.3	85	1.5
Fe²⁺	ND	7.6	ND	ND
Fe²⁺	638	11.0	86	1.5
Cu²⁺	725	10.0	133	1.8
UV	76	1.7	150	1.5

LDL was oxidized by dialysis against 5 μM FeSO₄ or CuSO₄ for 6–8 h, or by UV irradiation (see Materials and Methods for details on the oxidation procedures and the determinations of oxidation). ND, not determined; LPO, lipid peroxides.

had only weak stimulatory activity compared with the intact lipoprotein.

To explore the generality of these observations, LDL was oxidized by three different procedures—dialysis against FeSO₄, dialysis against CuSO₄, and ultraviolet irradiation. The oxidation level of all lipoproteins was measured as TBA-reacting substances as well as by lipid peroxides, fluorescence, and relative electrophoretic mobility (Table II). LDL that was extensively oxidized by incubation with Fe²⁺ or Cu²⁺ exhibited similar profiles in terms of all four oxidation parameters. Compared with the metal-oxidized lipoproteins, the UV-treated LDL was oxidized to a similar degree with respect to fluorescence and relative electrophoretic mobility, but much less oxidized in terms of lipid peroxides and TBA-reacting substances. The effect of these oxidized lipoproteins on EC migration is shown in Fig. 8. LDL oxidized by ultraviolet irradiation clearly did not inhibit EC migration, but
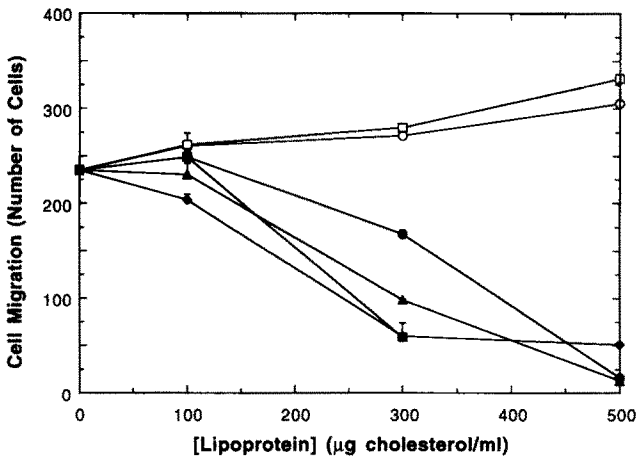

Figure 8. Antimigratory activity of LDL oxidized by different procedures. LDL was oxidized by incubation with 5 μM FeSO₄ or CuSO₄, or by UV irradiation for 4 h. EC migration was measured after treating the wounded cultures with lipoproteins for 24 h. Native LDL, TBARS = 0.2 nmol MDA/mg cholesterol (O); UV-oxidized LDL, TBARS = 1.7 nmol MDA/mg cholesterol (□); Fe-oxidized LDL, TBARS = 6.3 nmol MDA/mg cholesterol (●); Fe-oxidized LDL, TBARS = 7.6 nmol MDA/mg cholesterol (▲); Fe-oxidized LDL, TBARS = 11.0 nmol MDA/mg cholesterol (■); and Cu-oxidized LDL, TBARS = 10.0 nmol MDA/mg cholesterol (♦). Mean and standard error are shown.

rather it stimulated migration to at least the same extent as native LDL. Focusing on the results at 300 μg cholesterol/ml, a concentration that gave significant but submaximal inhibition, these results show that the inhibitory activity of all lipoproteins could be directly related to the oxidation level measured as TBA-reacting substances or lipid peroxides.

Discussion

We have used an in vitro "wound" model to investigate the effects of oxidized lipoproteins on the migration of bovine aortic EC. Our results show that oxidized LDL is a potent antimigration agent that reduces both the total number of migrating cells as well as the mean migration distance of these cells. The dose response of EC to oxidized LDL is biphasic, with a gradual increase in migration rate at concentrations up to 100 μg cholesterol/ml, followed by a steep decline to a nearly undetectable level of migration. The level of oxidation and the amount of lipoprotein required for half-maximal inhibitory activity are substantially higher than those reported for other effects on cultured EC, e.g., inhibition of PDGF production (21), inactivation of endothelial-derived relaxing factor (10), and induction of monocyte chemotactic protein (13), among others. This suggests that the effects of oxidized LDL on migration may be mediated by a different pathway than the others. Alternatively, the stimulation of migration at low concentrations of oxidized LDL may oppose the inhibitory activity at higher concentrations thereby raising the apparent half-maximal antimigratory activity.

The mechanism by which oxidized LDL exerts antimigratory activity is not known. However, the possibility that the inhibition is secondary to mortal cell injury was considered, since oxidized LDL has been shown to be toxic, particularly under proliferative conditions, to endothelial and other cultured cells (25, 31). Several lines of experimental evidence suggest that the inhibition of migration in this case is independent of the toxic effects. First, the antimigratory effects are reversible. Pretreatment of confluent EC with inhibitory concentrations of oxidized LDL reduced EC migration after its removal and subsequent wounding (Fig. 6). However, the inhibition was completely reversed in cultures wounded 16–24 h after removal of the oxidized LDL. Second, cells exposed to oxidized LDL did not die, as monitored by time lapse videomicrography, which showed that treated cells did not lift off the dish but rather moved more slowly than control cells. Exposure of cells to subtoxic concentrations of oxidized LDL results in the production of several injurious agents, such as the toxic oxysterols and oxidized fatty acid–derived aldehydes, including 4-hydroxynonenal (27, 54), and it cannot be ruled out that these agents may be responsible for the effects we observed.

Since oxidized LDL is known to alter the production and secretion of multiple growth factors and cytokines (21, 36, 41), we tested the idea that its antimigratory activity was due to decreased availability of basic FGF, a potent endogenous activator of EC migration (37). Exogenous addition of a maximally effective concentration of basic FGF, in the presence of oxidized LDL, did not restore EC migration to lipoprotein-free rates, indicating that alternate promigratory factors or distinct mechanisms pertain. The number of factors known to stimulate cell motility is large (for review see

reference 18). Several of these factors stimulate EC movement, including vascular endothelial growth factor (26) and scatter factor (48). However, an autocrine role for these factors in EC has not been established. Other mechanisms that should be considered include interference by the oxidized lipoproteins with intracellular signaling pathways, with the assembly of actin microfilaments and other structures required for motility, or with the secretion or activity of proteases that degrade extracellular matrix molecules and are required for cell movement. In support of the latter mechanism, Ohuchida et al. (42) have reported that oxidized lipids increase fibroblast production of two matrix metalloproteases, tissue collagenase and stromelysin, while decreasing the production of gelatinase.

A significant portion of the antimigratory activity was found in the lipid fraction of oxidized LDL. The lower potency of the extract, compared to the whole lipoprotein, may be due to loss of the inhibitory lipid species during extraction (as suggested by the decreased oxidation level), or to lower uptake of the lipid emulsion particles compared to the receptor-mediated uptake of the intact lipoprotein. Alternatively, a portion of the inhibitory activity may reside in oxidized apo B-100 fragments. Oxidized LDL contains numerous lipid oxidation products formed during the oxidative propagation reactions. The fact that the antimigratory activity is retained in the total lipid extract indicates that one or more of the new lipids is responsible; however, we have not yet determined their identities. Oxidized fatty acid products, such as 4-hydroxynonenal, can enhance the fluorescence of LDL at 360 excitation/430 emission (30) and can increase the net negative charge of the lipoprotein (56). The fact that among several oxidized LDL preparations, the antimigratory activity did not correlate as well with either of these two parameters as it did with total lipid peroxides (Table II) suggests that such long chain aldehydes may not be the mediators.

Oxidized lipoproteins appear to exist in arterial lesions in experimental animals and in humans (for review see reference 62). Several laboratories have demonstrated that antibodies recognizing lipid oxidation products (e.g., malondialdehyde, 4-hydroxy-nonenal) linked to proteins, and antibodies that bind unknown epitopes on oxidized LDL that are absent on native LDL, recognize epitopes present in arterial lesions (22). Furthermore, LDL-like extracts of arterial lesions have properties more similar to oxidized LDL than to native plasma LDL (14, 63). Perhaps most importantly, the antibodies noted above recognize these arterial "LDL" preparations (63). Hence, the presence of oxidized LDL in the subendothelial space invites the speculation that the antimigratory effects we observe may also slow endothelial repair in vivo. However, it has yet to be shown that the spectrum of lipids in LDL oxidized in vitro is also present in oxidized forms of LDL existing in vivo. Furthermore, the arterial wall concentrations of oxidized LDL and its constituent lipid oxidation products have not yet been determined.

The antimigratory activity of oxidized LDL may play a role in vivo by limiting or adversely affecting endothelial repair in developing atherosclerotic lesions, in the recovering arterial wall after balloon angioplasty, or in a reendothelializing arterial graft. Early studies on regeneration of endothelium have shown that deendothelialization of the rat aorta with a balloon catheter is followed by a reproducible series of events beginning with rapid platelet aggregation, followed by reendothelialization, and culminating in marked intimal thickening due to smooth muscle cell migration and proliferation within 4 wk (49). A strong correlation between the duration of endothelial denudation and the degree of intimal thickening has been reported; Haudenschild and Schwartz (23) showed that injured regions that were covered by regenerated endothelium within 7 d after injury were completely spared from intimal thickening. Thus, while the mechanism(s) by which confluent endothelium reduces or prevents intimal thickening has not been resolved, it appears that the inhibition of endothelialization may accelerate lesion formation.

In view of the protection afforded by an intact endothelium, it may be unfortunate that endothelial healing after injury is limited in duration and extent in most animal species (47). The minimal ingrowth of EC from vessel anastomoses onto synthetic vascular grafts suggests that a similar limitation exists in humans. The cause of the incomplete repair remains a critical and unresolved issue, and several mechanisms have been explored. Reidy (45) has shown that the SMC pseudointima that forms adjacent to the regenerating endothelium is not responsible for the cessation of growth. Inhibition of regeneration by fibronectin and other specific components of the extracellular matrix has also been considered (19). In testing this hypothesis, Lindner et al. (32) have shown that the presence of fibronectin on denuded vessel surfaces did not correlate with its regenerative capacity. However, other matrix components have not been as rigorously examined. Growth factors are likely to have a role in endothelial regeneration since several, most notably basic FGF and TGF-β, influence EC migration and proliferation in vitro. Recent reports suggest that injection of either acidic or basic FGF stimulates EC repair after denuding injury (6, 33). These data clearly demonstrate the importance of FGF in vascular repair but do not explain why regenerating cells reach a point where exogenous growth factors are required for further repair.

We could speculate from our observations that subendothelial oxidized lipids and lipoproteins may play a negative role in endothelial regeneration by inhibiting EC migration and perhaps proliferation. There are no data that directly implicate lipids, oxidized or otherwise, in the incomplete regeneration of endothelium after angioplasty. However, several laboratories have shown that the uptake and accumulation of lipid after balloon catheter injury (in normo- and hypercholesterolemic animal models) is greatest beneath the newly regenerated endothelium, and not in the adjacent, still-denuded intima (38, 60). One interpretation of these observations is that subendothelial lipoproteins may be oxidatively modified by cell-mediated processes (40, 56) and trapped due to the high affinity of oxidized LDL for collagen and other matrix molecules (28), and that these oxidized lipoproteins subsequently reduce reendothelialization. Further studies in organ culture and in animal models will be necessary to understand the processes involved in arterial oxidation of LDL, and to define the role that these lipoproteins play in EC regeneration.

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