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Oxidatively modified low density lipoproteins: A potential role in recruitment and retention of monocyte/macrophages during atherogenesis

(chemotaxis/atherosclerosis/scavenger receptor/endothelial cells/acetylated low density lipoprotein)

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ABSTRACT **Previous studies in this laboratory established that low density lipoprotein (LDL) incubated with cultured endothelial cells, smooth muscle cells, or macrophages undergoes a free radical-catalyzed oxidative modification that generates lipid peroxides and extensive structural changes in the LDL molecule. The oxidatively modified LDL strongly inhibited chemotactic responses of the mouse resident peritoneal macrophage. The present studies show that this oxidized LDL does *not* inhibit the motility of mouse monocytes and actually exhibits a chemotactic activity for human monocytes; the chemotactic activity of the oxidized LDL resides in the lipid fraction. These findings allow us to propose a pathogenetic sequence by which elevated plasma LDL levels, followed by oxidative modification in the arterial wall, could sufficiently account for the generation of the lipid-laden foam cells and the initiation of the fatty streak, the earliest well-defined lesion in atherogenesis.**

Accumulation of lipid in the arterial intima is central to the development of atherosclerosis. Intimal lipid accumulates intracytoplasmically in foam cells, which are derived both from medial smooth muscle cells (1, 2) and monocyte-derived macrophages (3–5), the latter probably being quantitatively more important (3–5). Exactly how monocytes are recruited and retained in the artery wall remains unclear, but probably the initial event is adhesion to the endothelial surface (3) followed by penetration that is influenced by a chemotactic factor(s). Many different factors chemotactic for monocytes have been described (6); the relative importance of these remains uncertain. Crude extracts of whole aorta contain chemotactic activity (7), and cultured arterial smooth muscle cells and macrophages release chemotactic activity into the culture medium (8). We recently described release of chemotactic activity for mouse resident peritoneal macrophages from cultured aortic endothelial cells (9) and showed that oxidatively modified low density lipoprotein (LDL) inhibited the chemotactic response of the macrophage. In the present studies we confirm the finding of Berliner *et al.* (10) that endothelial cell-conditioned medium is chemotactic also for human monocytes. However, oxidatively modified LDL, instead of *inhibiting* the motility of monocytes actually enhances their motility. We further show that the chemotactic activity resides in the lipid fraction of the modified LDL, presumably in one or another peroxidized lipid component. Thus oxidative modification of LDL, in addition to favoring the accumulation of cholesterol stores in developing foam cells (11), could play a role in recruitment and retention of monocyte/macrophages into the subendothelial space and, finally, may contribute to atherogenesis through injury to endothelial cells.

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MATERIALS AND METHODS

Ham's F-10 medium and fetal bovine serum were from HyClone (Logan, UT); female Swiss Webster mice were from Simonsen Laboratories (Gilroy, CA); Ficoll/Hypaque, bovine serum albumin, zymosan A, fucoidin, polyinosinic acid, and Hank's balanced salt solution were from Sigma.

Cells and Conditioned Media. The rabbit aortic endothelial cells (EC) were from a line established and characterized by Buonassisi and Venter (12). These cells were grown in Ham's F-10 medium/15% fetal bovine serum containing epidermal growth factor at 10 μg/ml in 60-mm plastic culture dishes and were used at confluence. EC were washed three times with serum-free medium and then incubated for 24 hr with 2.0 ml of Ham's F-10 medium at 37°C. After incubation, the conditioned medium was aspirated, and any detached cells were removed by low-speed centrifugation.

Resident mouse peritoneal macrophages were isolated from female Swiss Webster mice by lavage with phosphate-buffered saline as described previously (9).

Human and mouse monocytes were isolated by the method of Kumagai *et al.* (13) using Ficoll/Hypaque gradient centrifugation (14). Following the Ficoll/Hypaque centrifugation, the mouse mononuclear layer was heavily contaminated with platelets, which required extensive washing to remove—a problem not encountered during the isolation of human monocytes. The mononuclear cells were then resuspended in RPMI 1640/10% fetal bovine serum, plated on serum-treated 60-mm dishes, and incubated at 37°C for 2 hr. The nonadherent cells were removed by rinsing the plate with phosphate-buffered saline, then the adherent cells were detached by incubation in phosphate-buffered saline/5% fetal bovine serum/0.02% EDTA at 4°C for 30 min. The cells were then washed extensively and resuspended in Hank's balanced salt solution/0.2% bovine serum albumin at 2.0×10^6 cells per ml. The resultant cell suspension contained greater than 90% monocytes, as determined by nonspecific esterase staining, which were greater than 95% viable as determined by trypan blue exclusion.

Lipoproteins. LDL (ρ = 1.019–1.063) was isolated by ultracentrifugation from pooled normal human plasma collected in EDTA (1 mg/ml) (15). Protein was determined by the method of Lowry *et al.* (16) using bovine serum albumin as a standard. EC-modified LDL was prepared as previously described (9). Reisolated EC-modified LDL was obtained by flotation at ρ = 1.21 for 24 hr. Acetyl-LDL was prepared by reaction with acetic anhydride as described by Basu *et al.* (17). Copper-oxidized LDL was prepared by incubating LDL (100 μg/ml) with 5 μM Cu^{2+} in Ham's F-10 medium at 37°C for 24 hr (18). Lipid extractions were done by using $CHCl_3$/MeOH according to the method of Bligh and Dyer

Abbreviations: LDL, low density lipoprotein; EC, endothelial cell(s); CI, chemotactic index.

(19). The chloroform phase was evaporated under nitrogen, and the lipids were dissolved in 10 μl of EtOH, and then appropriate amounts were added to Ham's F-10 medium to correspond to the original concentration of intact LDL for the chemotaxis assay.

Chemotaxis Assay. Chemotaxis was measured in a 48-well modified Boyden micro chemotaxis chamber (Neuro Probe, Cabin John, MD) as described earlier (9). Assays were run in triplicate, using a 5-hr incubation for the macrophages or a 2-hr incubation for the monocytes. Chemotactic activity is expressed as chemotactic index (CI), defined as the number of cells migrating in response to the test substance divided by the number migrating when unincubated control medium was present in both chambers. Seven to eight grid areas were counted per sample, and averaged results shown are, in every case, representative of two or more replicate protocols.

RESULTS

Serum-free medium that had been conditioned by incubation with monolayers of rabbit aortic EC (EC-conditioned medium), shown previously to be chemotactic for mouse peritoneal macrophages (9), consistently also contained chemotactic activity for freshly isolated human monocytes. In 13 separate experiments the CI for the EC-conditioned medium averaged 6.1 ± 1.0 (mean ± SEM). This activity was resistant to boiling in the serum-free medium for 5 min. However, when 10% fetal bovine serum was added before boiling, the activity was lost, probably due to coprecipitation. The activity was not extracted into the lipid phase of a $CHCl_3$/MeOH extraction. When evaluated in a modified checkerboard assay, the EC-conditioned medium was both chemotactic and chemokinetic for human monocytes (Table 1).

We next evaluated the chemotactic responses of human monocytes to native LDL and modified forms of LDL. Native LDL added to control F-10 medium (not previously incubated with cells) had no intrinsic chemotactic activity (Table 2, Exp. 1). However, when native LDL was present throughout the conditioning incubation, the resulting conditioned medium (now containing *EC-modified LDL*) had a CI even higher than that of the medium conditioned without LDL (Table 2, Exp. 3). Subsequently, in 13 more experiments in which serum-free medium was conditioned in the presence of LDL, the monocyte CI was 11.2 ± 1.2 (mean ± SEM)—i.e., almost twice that found in the absence of added LDL. This implied that LDL either (*i*) enhanced the release of, or the effectiveness of, some chemotactic factor(s) or (*ii*) was itself intrinsically chemotactic. We reisolated EC-modified LDL by density ultracentrifugation and showed that it was, indeed, itself directly chemotactic for human monocytes (Table 2, Exp. 4). Thus the overall chemotactic response of monocytes to EC-conditioned medium containing EC-modified LDL probably represents an additive combination of the response to a chemotactic factor(s) released by EC and the response to the EC-modified LDL itself.

Table 1. Chemotactic and chemokinetic activities of EC-conditioned medium for human monocytes

Addition to lower chamber	Addition to upper chamber	CI*
Control medium	Control medium	(1.0)
EC-conditioned F-10 medium	Control medium	6.3 ± 0.8
EC-conditioned F-10 medium	EC-conditioned F-10 medium	2.8 ± 0.9
Control medium	EC-conditioned F-10 medium	0.8 ± 0.2

*Number of cells migrating in response to the test substance divided by number of cells migrating when control medium is present in both chambers. Results are expressed as mean ± SD; $n = 7$.

Table 2. Chemotactic activity of LDL and modified forms of LDL for human monocytes

Exp.	Sample	CI
1	Native LDL, 100 μg/ml, added to control F-10 medium	1.4 ± 0.2
2	EC-conditioned F-10 medium	5.5 ± 0.5
3	EC-conditioned F-10 medium prepared in the presence of LDL, 100 μg/ml	9.5 ± 0.5
4	Reisolated EC-modified LDL, 100 μg/ml, added to control F-10 medium	6.4 ± 0.5
5	Cu^{2+}-oxidized LDL, 100 μg/ml, added to control F-10 medium	6.1 ± 0.5

Results are expressed as mean ± SD; $n = 7$.

Further evidence that oxidatively modified LDL is itself chemotactic came from studies using LDL incubated in the absence of cells but in the presence of high concentrations of Cu^{2+}. Oxidatively modified LDL is cytotoxic (20, 21) and shares a number of characteristics with EC-modified LDL (18). As shown in Table 2, Exp. 5, Cu^{2+}-oxidized LDL was also chemotactic for human monocytes. Control F-10 medium containing Cu^{2+} but no LDL was without activity (data not shown). Furthermore, the data in Table 3 show that both EC-modified LDL and Cu^{2+}-oxidized LDL are primarily chemotactic with little chemokinetic activity.

Both EC-modified and Cu^{2+}-oxidized LDL are preferentially degraded by macrophages and, at least partially, by way of the same receptor—the acetyl-LDL receptor (18, 22). Because the monocyte contains or expresses smaller numbers of this receptor (23, 24), it seems *a priori* unlikely that the acetyl-LDL receptor is involved in the chemotactic response of monocytes toward oxidized LDL. Nevertheless, we looked for monocyte chemotactic responses to several compounds previously shown to be chemotactic for macrophages (acetyl-LDL, fucoidin, and polyinosinic acid), all of which presumably interact with the acetyl-LDL receptor (25). However, none of these agents had chemotactic activity for circulating human monocytes (data not shown). These results agree with those recently reported by Haberland *et al.* (26).

To define the chemical nature of the chemotactic component in modified LDL, the lipids from EC-modified LDL and from Cu^{2+}-oxidized LDL were extracted using $CHCl_3$/MeOH. As shown in Table 4, the extracted lipids were strongly chemotactic when derived from modified LDL (either EC-modified or Cu^{2+}-oxidized), while the lipids extracted from native LDL had no effect. Thus the chemotactic activity resides predominantly in one or more of the peroxidized lipid components. However, the possibility that other components may contribute in some measure to the chemotactic activity of the intact modified LDL cannot be dismissed.

The described studies were done with freshly isolated *human* monocytes, whereas our previous studies were done with *mouse* resident peritoneal macrophages (9). We next

Table 3. Chemotactic and chemokinetic activities of EC-modified and Cu^{2+}-oxidized LDL for human monocytes

Addition to lower chamber	Addition to upper chamber	CI
Control medium	Control medium	(1.0)
EC-modified LDL	Control medium	8.5 ± 0.6
EC-modified LDL	EC-modified LDL	1.9 ± 0.4
Control medium	EC-modified LDL	1.3 ± 0.3
Cu^{2+}-oxidized LDL	Control medium	8.3 ± 0.8
Cu^{2+}-oxidized LDL	Cu^{2+}-oxidized LDL	0.7 ± 0.2
Control medium	Cu^{2+}-oxidized LDL	0.4 ± 0.1

LDL was added at 100 μg/ml in control F-10 medium. Results are expressed as mean ± SD; $n = 7$.

Table 4. Chemotactic activity of lipids extracted from EC-modified and Cu^{2+}-oxidized LDL

Sample	CI
Native LDL, 100 μg/ml*	1.6 ± 0.5
Lipids extracted from native LDL†	1.1 ± 0.3
EC-modified LDL, 100 μg/ml	9.5 ± 0.5
Lipids extracted from EC-modified LDL†	8.7 ± 0.5
Cu^{2+}-oxidized LDL, 100 μg/ml*	8.0 ± 0.5
Lipids extracted from Cu^{2+}-oxidized LDL†	9.4 ± 0.5

*LDL added in control F-10 medium.
†Lipids extracted using $CHCl_3$/MeOH and added back in ethanol as described under *Materials and Methods*. Results are expressed as mean ± SD; $n = 7$.

compared the responses of circulating mouse monocytes, circulating human monocytes, and mouse resident peritoneal macrophages to determine whether the observed differences were due to differences in cell type or in species. The data are summarized in Table 5. Both human and mouse monocytes responded similarly to EC-conditioned medium (Exp. 1), and this response was not inhibited by the presence of oxidatively modified LDL (EC-modified LDL, Exp. 2). In contrast, the chemotactic response of mouse macrophages to EC-conditioned medium (Exp. 1) was strongly inhibited by the presence of oxidatively modified LDL (either EC-modified or Cu^{2+}-oxidized LDL) (Exp. 2). Neither human nor mouse monocytes showed chemotactic responses to acetyl-LDL, fucoidin, or polyinosinic acid. But in confirmation of our previous findings (9) mouse peritoneal macrophages showed chemotactic responses to all three substances. The circulating mouse monocyte differed from the human monocyte in that it did not show a positive chemotactic response to oxidatively modified LDL although its motility was not inhibited. This deserves further investigation, however, because of difficulties in isolating mouse monocytes caused by a tendency of platelets to adhere tightly to the monocytes, as mentioned above. Generally, the differences noted appear to be the differences between a circulating monocyte and a resident macrophage—a cell-type difference.

DISCUSSION

The unexpected and potentially most important finding in these studies was that oxidatively modified LDL, previously shown to inhibit the chemotactic response of resident macrophages (9), is actually chemotactic for the circulating monocyte. Thus, the generation of oxidized LDL in the arterial wall could play a dual role. Initially it might favor monocyte recruitment (Fig. 1, I). Presumably the monocytes must first adhere to the endothelium. As mentioned, Gerrity *et al.* (7) have reported that hypercholesterolemia itself favors monocyte adhesion, although the specific role of LDL is unclear. Later, after phenotyptic alteration of the monocyte to that of a tissue macrophage, oxidized LDL would inhibit its motility and thus its probability of exiting from the vessel wall (Fig. 1, II). Our previous studies showed that the rate of uptake of oxidatively modified LDL, via the acetyl-LDL receptor, is sufficiently great to convert macrophages to foam cells, as indicated in Fig. 1, III. Finally, as shown by others, the oxidatively modified LDL has the potential of damaging endothelial cells (20, 21). Such endothelial injury (Fig. 1, IV) might further enhance the rate of LDL penetration into the vessel—either through actual breaks in the lining monolayer or as a result of modification in trans-endothelial cell transport functions. Recent studies by Schwenke and Carew (27) have provided further evidence that the rate of uptake and degradation of LDL at lesion-prone areas is greater than that in lesion-resistant areas even in the normal rabbit, and the differences, of course, become progressively more exaggerated as the lesions develop. The overall hypothesis suggests four effects of oxidatively modified LDL that could favor the atherogenic process. If operative *in vivo*, these mechanisms could explain the generation of the fatty streak as being initiated by elevated LDL levels. This would increase LDL penetration into the subendothelial space where oxidation of LDL would generate the four processes schematized in Fig. 1. Because the macrophage itself can oxidatively modify LDL, the stage for autocatalytic progression is set. To what extent do these phenomena demonstrated in cell culture occur *in vivo*?

The presence of lipid peroxides has been reported in circulating plasma (28) and, at an even higher level, in atherosclerotic lesions (29, 30). Whether the levels are sufficient to evoke the mechanism proposed here remains to be determined. Various modified forms of LDL in the artery wall have been reported (31, 32), and it has also been reported that the LDL in inflammatory fluids shares a number of properties with oxidatively modified LDL (33). However, the hypothesis that oxidized LDL plays a role in recruitment and retention of arterial wall macrophages will require critical testing *in vivo*.

The present studies also demonstrate the production by an established line of rabbit aortic EC of a chemotactic and chemokinetic factor (or factors) for human monocytes. Similar chemotactic activity was demonstrated in conditioned medium from human umbilical vein and bovine endothelial cells (data not shown). These results generally agree with those of Berliner *et al.* (10), who recently described monocyte chemotactic activity produced by primary cultures of normal large vessel endothelial cells. However, that factor differed from the activity described here in that the former lacked chemokinetic activity, did not show enhanced activity when LDL was present during the conditioning incubation, and was heat labile. The latter may only reflect differences between heating with and without serum. Furthermore, the lack of enhanced activity when LDL is present depends on oxidative modification of the LDL, and the serum in the

Table 5. Chemotactic responses of mouse monocytes, human monocytes, and mouse macrophages

Exp.	Sample	CI: Mouse macrophage	CI: Mouse monocyte	CI: Human monocyte
1	EC-conditioned F-10 medium	4.6 ± 0.3	5.5 ± 0.9	4.1 ± 0.1
2	EC-conditioned F-10 medium prepared in the presence of LDL, 100 μg/ml	0.6 ± 0.1	5.3 ± 0.6	9.0 ± 0.9
3	Cu^{2+}-oxidized LDL, 100 μg/ml	0.1 ± 0.1	0.9 ± 0.2	8.4 ± 0.6
4	Acetyl-LDL, 100 μg/ml	4.7 ± 0.3	ND	1.6 ± 0.2
5	Fucoidin, 50 μg/ml	7.0 ± 0.6	1.2 ± 0.2	1.0 ± 0.2
6	Polyinosinic acid, 50 μg/ml	11.4 ± 1.0	1.1 ± 0.2	1.4 ± 0.2

ND, experiment not done. All samples were prepared at the appropriate concentrations in control F-10 medium. Results are expressed as mean ± SD; $n = 7$.

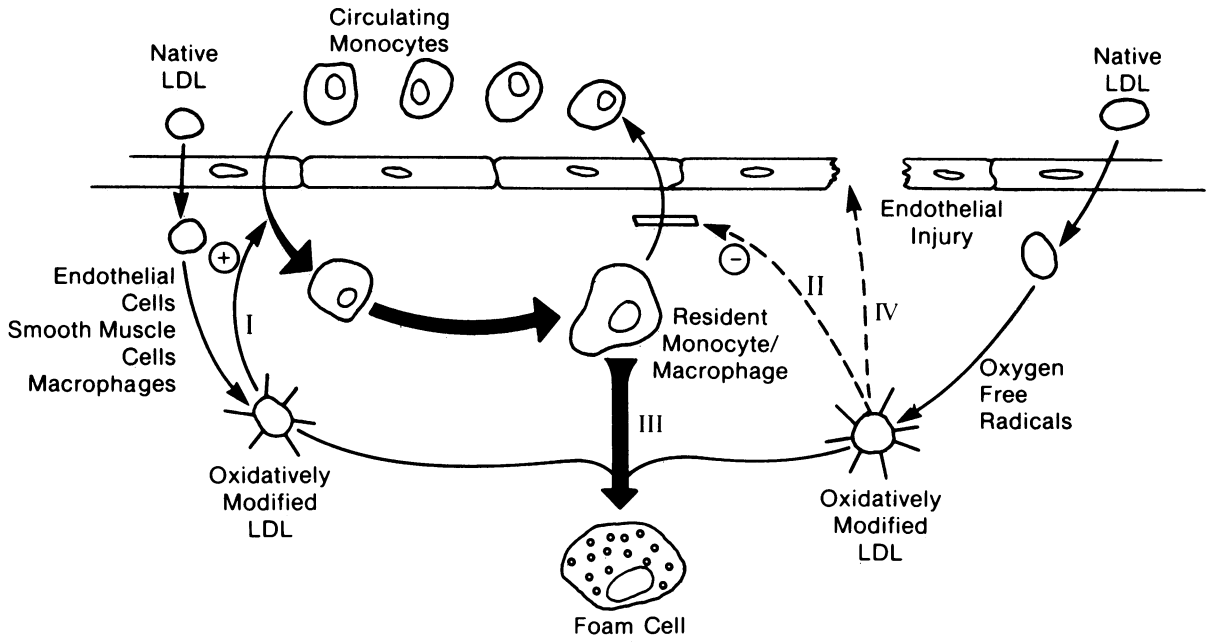

FIG. 1. Schematic of a hypothesis that could explain how a high plasma concentration of LDL, by increasing the generation of oxidatively modified LDL in the subendothelial space, could account for the initiation of atherogenesis and the formation of the fatty streak.

studies of Berliner *et al.* would have prevented such modification.

An unresolved question is whether the differences between the human monocyte and the human *arterial* macrophage are similar to the differences between the mouse monocyte and the mouse peritoneal macrophage. Table 5 suggests that the human monocyte and the mouse monocyte share properties and that both stand in contrast to the tissue macrophage. However, the mouse monocyte, while it was not inhibited by oxidized LDL, failed to show a positive chemotactic response; this may reflect a true difference in cellular response between the two species. However, because of platelet adherence during preparation, these mouse cells had to be centrifuged and washed more extensively than the human monocytes. Thus, the apparent difference may only reflect a difference related to the isolation technique for the mouse monocytes. However, the fact that oxidatively modified LDL is chemotactic for circulating human monocytes is clearly established by these studies.

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