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H F McMurray, … , S Parthasarathy, D Steinberg

Research Article

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Oxidatively Modified Low Density Lipoprotein Is a Chemoattractant for Human T Lymphocytes

Heather F. McMurray, Sampath Parthasarathy, and Daniel Steinberg
Department of Medicine, University of California, San Diego, La Jolla, California 92093

Abstract

Oxidatively modified low density lipoprotein (Ox-LDL) is a known chemoattractant for monocytes. Here we demonstrate, using a modified Boyden chamber assay, that human peripheral blood T lymphocytes, but not B lymphocytes, also respond chemotactically to Ox-LDL, showing a threefold increase over control and an optimum response at 10 μg/ml. Copper and endothelial cell–oxidized LDL and β-VLDL were used and gave similar results. The activity was not chemokinetic and native LDL possessed no chemoattractant activity. The chemoattractant activity was found to reside in the lipid fraction of Ox-LDL. Lysophosphatidylcholine is a major phospholipid component of Ox-LDL and is known to be chemotactic for monocytes. We show that lysophosphatidylcholine is also chemotactic for T lymphocytes with a maximal fourfold increase at 10 μM. Nonmetabolizable analogues of lysophosphatidylcholine had no such chemotactic effect. Thus, Ox-LDL, by virtue of its lysophosphatidylcholine content, may contribute to the recruitment of both T lymphocytes and monocytes into developing atherosclerotic lesions. (*J. Clin. Invest.* 1993. 92:1004–1008.) Key words: chemotaxis • B lymphocytes • lysophosphatidylcholine • β-VLDL • atherosclerosis

Introduction

It has been recognized for some time that one of the earliest events in atherosclerotic lesion formation is the intimal accumulation of monocyte-derived macrophages and their subsequent development into foam cells (1–3). Only recently, however, has the presence of substantial numbers of T lymphocytes been demonstrated in human fatty streaks, fibrous plaques, and advanced plaques (4–8). Within the fibrous cap of human atherosclerotic lesions, T cells can represent up to 20% of the total cell population, while B lymphocytes or natural killer cells are rare (4).

Studies in the cholesterol-fed rabbit model of atherosclerosis have been used to determine the sequence of lymphocyte and monocyte entry into the vessel wall. After only 1 wk of cholesterol feeding, both monocytes and T lymphocytes were found adhering to the aortic surface and accumulating within the lesion (9). Finding that the infiltration of these two inflammatory cell types occurs at about the same time raises the

possibility that they are responding to a common stimulus. While a number of chemoattractants have been suggested for the recruitment of monocytes, little is known about the factors that may influence T lymphocyte recruitment. Previous studies from this laboratory demonstrated that oxidized LDL (Ox-LDL)[1] is a potent chemoattractant for monocytes, but not for granulocytes. It was shown that lysophosphatidylcholine (lyso-PtdCho), generated during the oxidation of LDL, was the major component responsible (10, 11). These studies show that Ox-LDL, but not native LDL, is also a chemoattractant for circulating T lymphocytes. Furthermore, lyso-PtdCho was chemotactic for T cells, while neither Ox-LDL nor lyso-PtdCho showed any chemotactic activity for B lymphocytes. Thus, lyso-PtdCho, generated during oxidation of LDL or from the membrane phospholipids of damaged cells, could play a pivotal role in the recruitment of both monocytes and T lymphocytes not only into atherosclerotic lesions but also to sites of tissue injury and repair generally.

Methods

Materials. Ficoll/Hypaque, BSA, HBSS, dioctanoyl, and Ham's F10 medium were from Sigma Chemical Co. (St. Louis, MO); RPMI 1640 medium was from Irvine Scientific (Santa Ana, CA); nylon wool was from Polysciences Inc. (Warrington, PA); FCS was from Hyclone Laboratories (Logan, UT); and micro-chemotaxis chambers and 5-μm filters were from NeuroProbe (Cabin John, MD).

Cells. Human T lymphocytes were isolated from normal human peripheral blood by the method of Julius et al. (12). Briefly, mononuclear cells were recovered from the interface after Ficoll/Hypaque gradient centrifugation, resuspended in RPMI 1640/10% human autologous serum, and plated. After a 2-h incubation, nonadherent cells were collected by rinsing plates with serum-free RPMI 1640. This nonadherent cell population was then enriched for T cells using a nylon wool column and the nylon wool nonadherent T cells were recovered. At this point adherent B cells were recovered from the column with ice-cold medium. T cells were resuspended in HBSS/0.2% BSA at 10×10^6 and B cells at 2×10^6 cells/ml for use in chemotaxis assays. The nonadherent cell suspension eluted from the column contained 88% T lymphocytes as determined by FACS® analysis using a monoclonal antibody, Leu 4, which is directed against CD3 (Becton Dickinson and Company, Mountain View, CA), 7% B cells using goat anti–human IgG (Sigma Chemical Co.) and 0% monocytes using an antibody against CD14 (Becton Dickinson and Company). Further FACS® analysis of isolated T cell populations for CD4 and CD8 positive cells showed an average CD4/CD8 ratio of 0.9. Anti-CD4 and CD8 monoclonal antibodies were purchased from Beckton Dickinson and Company.

Rabbit aortic endothelial cells were from a line established and characterized by Buonassisi and Venter (13). These cells were maintained in Ham's F10 medium/15% FCS supplemented with epidermal growth factor at 10 μg/ml and used at confluence.

Address correspondence to Dr. Sampath Parthasarathy, UCSD Department of Medicine, 0682, 9500 Gilman Drive, La Jolla, CA 92093-0682.

1. *Abbreviations used in this paper:* CI, chemotactic index; lyso-PtdCho, lysophosphatidylcholine; MDA, malondialdehyde; Ox-LDL, oxidized low density lipoprotein; TBARS, thiobarbituric acid substance.

Lipoproteins. LDL (density = 1.019–1.063 g/ml) was isolated by ultracentrifugation from pooled normal human plasma collected in EDTA (1 mg/ml) (14). Protein was determined by the method of Lowry et al. (15) using BSA as a standard. Copper-oxidized LDL was prepared by incubating LDL, at a concentration of 100 μg/ml, with 10 μM Cu^{2+} in F10 medium at 37°C for 24 h. Endothelial cell–modified LDL was prepared as previously described, using cultured rabbit aortic endothelial cells (16). β-VLDL was prepared from the plasma of cholesterol-fed New Zealand White rabbits and VLDL from non–cholesterol-fed rabbits as previously described (17).

Lipid peroxidation. The extent of lipid peroxidation was measured as thiobarbituric acid reactive products (TBARS) and expressed as malondialdehyde (MDA) equivalents. Both native and β-VLDL routinely had < 5 nmol MDA/mg protein, while the content of MDA in Ox-LDL samples (copper and endothelial cell–oxidized LDL) ranged from 50 to 70 nmol/mg protein.

Lipid extractions. Lipid extractions were carried out according to the method of Bligh and Dyer (18). The chloroform phase was evaporated under nitrogen, the lipids were redissolved in ethanol, and aliquots were then added to HBSS for assessment in the chemotaxis assays.

Phospholipid analysis. Lipids were extracted from lipoproteins (200 μg of protein) with chloroform/methanol and the solvents were evaporated under nitrogen. The phospholipids were separated by TLC on silica gel G with chloroform/methanol/water, 65:35:6 (vol/vol/vol) and visualized with iodine vapor. Phosphatidylcholine and lyso-PtdCho were scraped from the plates and assayed for phosphorus content (19).

Lyso-PtdCho and analogues. 1-Palmitoyl-sn-glycerol-3-phosphocholine (lyso-PtdCho) was purchased from Sigma Chemical Co. The lyso-PtdCho analogues, 1-palmityl-propanediol-3-phosphocholine, 1-palmityl-ethanediol-2-phosphocholine, and 1-palmitoyl-ethanediol-2-phosphocholine were a kind gift of Dr. W. J. Baumann (Hormel Institute, Austin, MN), prepared according to Murari et al. (20) and analyzed for purity using nuclear magnetic resonance spectroscopy. Analysis by TLC using a solvent system of $CHCl_3/CH_3OH/H_2O$ (65:35:6, vol/vol/vol) indicated that all samples were pure and migrated as a single species. Samples were dried under N_2 and resuspended in HBSS at appropriate concentrations for the chemotaxis assay.

Chemotaxis assay. Chemotaxis was measured in a 48-well modified Boyden microchemotaxis chamber (NeuroProbe) as described previously (16). Assays were carried out in triplicate, using a 5-μm membrane. T lymphocytes were suspended in HBSS at 10×10^6 cells/ml and incubated at 37°C in a 5% CO_2 atmosphere for 3 h. The filters were dehydrated and stained (LeukoStat; Fisher Diagnostics, Pittsburgh, PA) and then mounted on glass slides. The cells that had migrated to the underside of the filter were counted. Seven grid areas of 0.25 mm^2 each were counted per sample. Chemotactic activity is expressed as a chemotactic index (CI), defined as the number of cells migrating in response to the test substance divided by the number migrating when control medium was present in both upper and lower chambers. The mean results are shown and are, in every case, representative of two or more replicate protocols.

Results

Effect of oxidized LDL on T lymphocyte chemotaxis. Ox-LDL was prepared either by incubation with copper ion or by incubating overnight with a monolayer of endothelial cells. As shown in Fig. 1, both forms of Ox-LDL were chemotactic for T lymphocytes, with more than a threefold increase in the number of migrating cells at a concentration of 10 μg/ml. The decrease in response at higher concentrations of Ox-LDL has ample precedent in the behavior of other chemoattractants and was not due to toxicity as determined by trypan blue exclusion after a 3-h incubation of T lymphocytes with up to 50 μg/ml
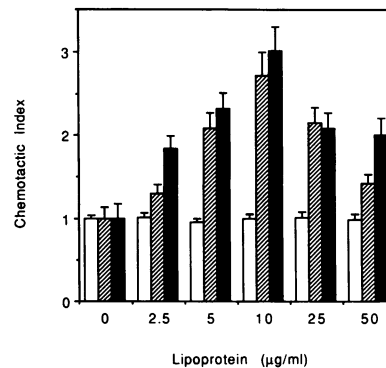

Figure 1. Human T lymphocyte chemotactic response to native (□), copper-oxidized (▨), and endothelial cell-oxidized (■) LDL. Samples were diluted in HBSS before testing in chemotaxis assay. Medium conditioned by endothelial cells in the absence of LDL served as the control for endothelial cell-oxidized LDL, while medium without LDL served as the control for native and copper-modified LDL. The CI for each sample is expressed as mean±SD (seven fields counted). Results are representative of those from three separate experiments.

Ox-LDL. Native LDL had no chemotactic activity over the same concentration range. Checkerboard analysis was used to distinguish chemotaxis from chemokinesis and demonstrated that Ox-LDL was exclusively chemotactic in the range of 2.5–25 μg/ml (Table I).

Effect of Ox-LDL on B lymphocyte chemotaxis. As shown in Table II, Ox-LDL in the concentration range 0–100 μg/ml had no significant chemoattractant activity for peripheral B lymphocytes.

The lipid components of Ox-LDL and lyso-PtdCho contain chemotactic activity. Unfractionated lipids extracted from Ox-LDL, but not from native LDL, were found to contain chemotactic activity for T lymphocytes, inducing the same degree of chemotactic response seen with intact Ox-LDL (data not shown). A previous report from this laboratory established that the lyso-PtdCho component of Ox-LDL was responsible for much of the ability of Ox-LDL to attract circulating monocytes (11). Consequently, lyso-PtdCho was tested using T lymphocytes as the target cells. Fig. 2 shows a fourfold increase in T lymphocyte migration in the presence of 5–10 μM lyso-Ptd-Cho. This concentration lies well below the critical micellar concentration for lyso-PtdCho in HBSS and so is not due to a simple detergent effect. Furthermore, the effective concentration of lyso-PtdCho correlates well with the estimated lyso-Ptd-Cho content of the effective concentration of Ox-LDL. Thus, 10 μg of Ox-LDL contains ∼ 2.5 nmol of lyso-PtdCho. In contrast, PtdCho possessed no chemoattractant activity for T lymphocytes.

β-VLDL is chemotactic for T lymphocytes. In the cholesterol-fed rabbit model of atherosclerosis, the major lipoprotein fraction is β-migrating very low density lipoprotein (β-VLDL). We have previously demonstrated that β-VLDL is chemotactic for monocytes, probably by virtue of its high lyso-PtdCho content (17). The β-VLDL preparations contained, on average, 2.4 nmol of lyso-PtdCho/10 μg protein. Here, we show that β-VLDL is also chemotactic for T lymphocytes (Fig. 3). The maximal response was seen at 10 μg/ml and the magnitude of the response was comparable to that observed for Ox-LDL.

Extent of oxidative modification, lyso-PtdCho content, and the ability of modified LDL to stimulate T cell migration. Table III clearly shows the increase in production of chemoattractant agent(s) with time during incubation of native LDL with 5 μM copper acetate. The generation of chemotactic activity was

Table I. Checkerboard Analysis of the Chemoattractant Effect of Ox-LDL on Human T Lymphocytes

Cu Ox LDL below filter	Cu Ox-LDL above filter (µg/ml)				
	0	2.5	5	10	25
µg/ml					
0	1.0±0.09	0.93±0.08	1.06±0.10	1.02±0.10	1.04±0.13
2.5	1.29±0.11	1.07±0.11			
5	2.08±0.19		1.04±0.16		
10	2.73±0.26			1.03±0.11	
25	2.15±0.19				1.02±0.12

The CI for each sample is expressed as mean±SD (seven fields counted per sample). Results are representative of those from two separate experiments.

maximal after 8 h when the formation of lyso-PtdCho also reached its plateau, suggesting a parallel between chemoattractant production and lyso-PtdCho formation.

Lyso-PtdCho analogues and their chemotactic activity. Previous studies have suggested the potential involvement of a phospholipase C–type reaction in the monocyte chemotactic activity of lyso-PtdCho (21). To ascertain whether metabolizable analogues of lyso-PtdCho would have effects similar to those of lyso-PtdCho itself, several analogues of lyso-PtdCho were studied: palmitoyl-ethanediol-2-phosphocholine, palmityl-ethanediol-2-phosphocholine, and palmityl-propanediol-2-phosphocholine. The first is a metabolizable analogue; the latter two having the palmitate in ether linkage rather than ester linkage are not metabolizable. Fig. 4 clearly shows that only the metabolizable analogue (palmitoyl-ethanediol-2-phosphocholine) is chemotactic for T lymphocytes, showing a threefold increase in the 2.5–5 µM concentration range. The increase in the number of migrating cells induced by the metabolizable analogue is equivalent to that induced by 5 µM lyso-PtdCho in the same assay. The fact that only the metabolizable analogue possessed chemoattractant activity suggests that the lyso-PtdCho must first be taken up by the cells before exerting its effect and provides further evidence against this being a simple detergent effect.

Table II. Ox-LDL Is Not Chemotactic for Human B Lymphocytes

Ox-LDL	CI±SD
µg/ml	
0	1.00±0.10
2.5	0.93±0.11
5	0.94±0.14
10	0.89±0.16
25	1.00±0.10
50	0.89±0.09
100	0.90±0.10

The CI for each sample is expressed as mean±SD (seven fields counted per sample). Dioctanoyl, a protein kinase C activator, was used as a positive control to ensure that the B cells were capable of responding chemotactically. 100 µM dioctanoyl gave a chemotactic index of 2.82±0.15. Results are representative of those from two separate experiments.

Discussion

The demonstrated chemotactic activity of Ox-LDL for T lymphocytes suggests yet another mechanism by which Ox-LDL may influence circulating leukocytes to enter the intimal space. Native LDL had no such chemotactic activity. Furthermore, the fact that Ox-LDL was not chemotactic for isolated peripheral B lymphocytes is consistent with the observation that B lymphocytes are not found at any stage of lesion development (4, 22). Thus, it is entirely possible that Ox-LDL, generated in the subendothelial space, may be important in the very earliest stages of atherogenesis.

T cells persist in lesions at all stages (6, 8). They are especially prominent at the shoulders of more advanced lesions, in close association with macrophages, which are also present in high numbers in the shoulder regions (4, 7). The chemotactic effect described here may, therefore, be relevant not only in the initial stages of lesion formation but throughout the history of the lesion. IL-1 has been demonstrated to be a chemoattractant for T cells (23, 24) and Ox-LDL has been shown to increase the secretion of IL-1 (25, 26). Thus, there could be an indirect facilitation of T cell recruitment by Ox-LDL in addition to the direct effect demonstrated here.

It is possible that the chemotactic activity of Ox-LDL and β-VLDL for T lymphocytes could be accounted for by its content of lyso-PtdCho, in consonance with our earlier findings regarding the chemotactic activity of Ox-LDL for monocytes (10, 11). Furthermore, the increase in extent of modification over time paralleled with the observed increase in lyso-PtdCho
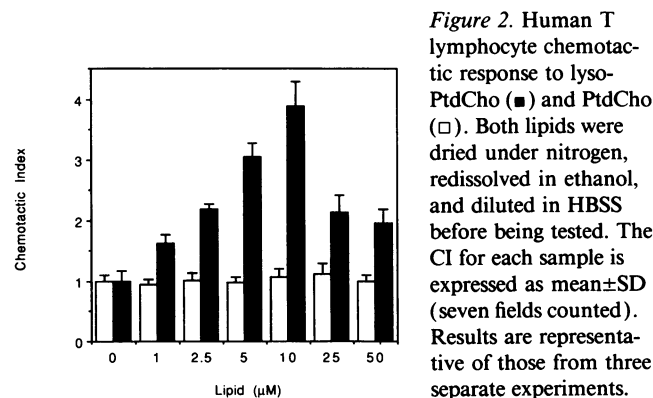
Figure 2. Human T lymphocyte chemotactic response to lyso-PtdCho (■) and PtdCho (□). Both lipids were dried under nitrogen, redissolved in ethanol, and diluted in HBSS before being tested. The CI for each sample is expressed as mean±SD (seven fields counted). Results are representative of those from three separate experiments.
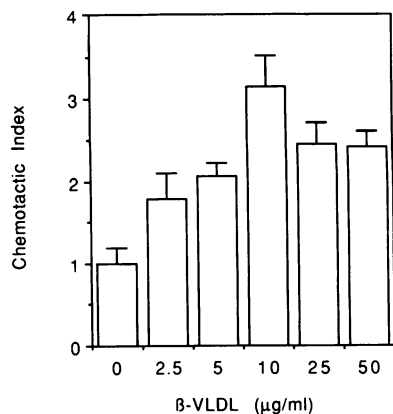
Figure 3. β-VLDL is chemotactic for human T lymphocytes. β-VLDL contained 2.4 nmol lyso-PtdCho/10 μg protein and was diluted in HBSS before being tested in the chemotaxis assay as described in Methods. Results are representative of those from two experiments.

Figure 4. Human T lymphocyte response to structural analogues of lyso-PtdCho: Palmityl-ethanediol-2-phosphocholine (□), palmityl-propanediol-3-phosphocholine (▨), and palmitoyl-ethanediol-2-phosphocholine (■) were dried under nitrogen and dissolved in HBSS before being tested for chemotactic activity. The CI for each sample is expressed as mean±SD (seven fields counted). 5 μM lyso-PtdCho was used as a positive control in each experiment. Results are representative of those from two separate experiments.

content and with the production of chemotactic activity for T lymphocytes. It has been previously reported that lyso-PtdCho is a chemoattractant for a murine thymic cell line and for concanavalin A–stimulated mouse spleen thymocytes (27). The concentrations of lyso-PtdCho required, however, were 20-fold higher than the effective concentrations in the present studies and lyso-PtdCho was not effective for resting thymocytes. The reasons for the differences are not clear. Also of interest is the finding that concentrations of lyso-PtdCho increase in the artery wall early in the formation of the lesion (28). Recent work from Dr. Michael Gimbrone's laboratory has shown that lyso-PtdCho can induce the expression of an endothelial cell adhesion molecule, VCAM-1, on endothelial cells (29). VCAM-1 is known to be involved in T lymphocyte adherence to endothelial cells (30) via a CD18-independent mechanism, and thus lyso-PtdCho may be involved in both the increased adherence and recruitment of T lymphocytes at sites of lesion formation.

In these studies, as in our previous studies of the monocyte chemotactic response (21), nonmetabolizable analogues of lyso-PtdCho were not chemotactic for T lymphocytes. This may reflect the need for further metabolism of the lyso-PtdCho in order for it to exert its chemotactic action. Alternatively, it may be that the concentration of nonmetabolizable analogues builds up in the plasma membrane and thereby erases any chemotactic gradient. Indeed, cells preincubated with the nonme-

tabolizable analogues showed a significantly reduced ability to respond chemotactically to lyso-PtdCho (data not shown).

The role of the T lymphocyte in atherosclerotic lesion development has yet to be established. Recent studies show that the T cells in the lesion are polyclonal (31) and that they are in an activated state (22, 32). It is not clear, however, if they are recruited in an activated state or are activated locally in the lesion. Our laboratory, however, has recently demonstrated the presence of immune complexes in the artery wall, providing at least one mechanism for the local activation of T cells (Ylä-Herrtuala, S. W. Palinski, S. W. Butler, S. Picard, D. Steinberg, and J. L. Witztum, manuscript submitted for publication). Once activated, T cells are a source of a wide range of lymphokines with the potential for myriad effects on the other cellular components of the lesion (33). Activated T cells are one of the few sources of IL-4, one of the few agents capable of inducing the expression of 15-lipoxygenase in monocyte/macrophages (34). There are several lines of evidence suggesting that the oxidation of LDL by macrophages and endothelial cells can be attributed, in part, to the activity of 15-lipoxygenase (35). The evidence includes the demonstration that the gene for 15-lipoxygenase is actively expressed in macrophage-rich areas of diffuse intimal thickening (36). IL-4 is chemotactic for macrophages (37). If it is also chemotactic for monocytes, there could be an interaction in which T cells help recruit additional macrophages, and vice versa. Furthermore, lymphocytes themselves have recently been shown capable of oxidizing LDL (38). Thus, there could be an important interplay between T lymphocytes and macrophages, accelerating both the oxidative modification of LDL and the recruitment of additional T lymphocytes and monocytes.

While there are, as just outlined, several ways in which T lymphocyte–macrophage interactions might be proatherogenic, the situation is decidedly more complicated. This became clear from recent in vivo studies. Rats depleted of T lymphocytes, either athymic rats or normal rats injected with a monoclonal antibody against T cells, developed larger proliferative lesions after balloon injury than did control rats with normal T cell populations (39). This suggests an antiatherogenic role for T lymphocytes. In contrast, rats injected with cyclosporin A, an inhibitor of T cell activation, were found to de-

Table III. Relationship between Extent of Oxidative Modification of LDL, LysoPC Content, and Chemotactic Properties for T lymphocytes

Time	TBARS	Lyso-PtdCho	CI
h	nmol MDA/mg protein	nmol/10 μg protein	mean±SD
0	0	1.0	1.08±0.12
2	2	1.39	1.26±0.13
4	10.9	1.67	2.33±0.27
8	23.17	2.08	4.23±0.54
24	58.96	2.61	4.21±0.23

Native LDL was oxidatively modified at a concentration of 100 μg/ml with 5 μM copper acetate for the times indicated. These samples were then assessed at 10 μg/ml for their ability to chemotactically attract human T lymphocytes. In addition, these samples were assayed for content of TBARS, given as nanomoles MDA equivalents per milligram protein, and for lyso-PtdCho content. Values shown are means of three separate experiments.
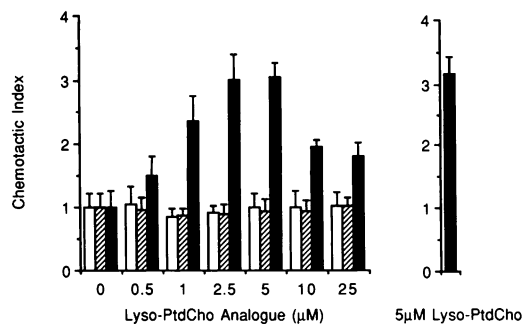
velop smaller lesions after balloon injury (40). It is not clear, however, if the cyclosporin A itself had a direct effect on other components of the lesion. Thus, T cells could be, in principle, either proatherogenic or antiatherogenic. Only further in vivo experiments will be able to assign relative importance to the several possibilities suggested by studies in vitro.

Acknowledgments

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