Mildly oxidized low-density lipoproteins suppress the proliferation of activated CD4⁺ T-lymphocytes and their interleukin 2 receptor expression *in vitro*

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Activated T-lymphocytes are present in early atherosclerotic lesions where they may interact with oxidized low-density lipoproteins (oxLDLs). In this study the non-specific effect of oxLDLs on the activation of T-cells *in vitro* was investigated. LDLs were oxidized by UV irradiation and characterized by a low level of lipid peroxidation and only slight apolipoprotein B modification. Peripheral blood lymphocytes from normal individuals were stimulated *in vitro* with the polyclonal activator phytohaemagglutinin in the presence of various doses of LDLs and oxLDLs. LDLs enhanced the proliferation of peripheral blood lymphocytes at doses up to 100 µg/ml but were inhibitory at 200 µg/ml, whereas low doses of oxLDLs (over 10 µg/ml) inhibited the proliferation. OxLDLs also inhibited the proliferative responses of an alloreactive CD4⁺ T-cell line immortalized by Herpes virus saimiri and an influenza haemagglutinin-

specific CD4⁺ T-cell clone. Viability tests using Trypan Blue exclusion or expression of Apo2.7, an apoptosis marker, did not indicate any significant cell death at doses up to 100 µg/ml oxLDL. At this concentration, cell-cycle analysis showed an accumulation of cells at the G₁/S interface in the CD4⁺ cell clone, without significant DNA fragmentation. The expression of the activation antigen CD25 on T-lymphocytes (on phytohaemagglutinin-activated T-cells and on CD4⁺ T-cell clone), requisite to the commitment of activated T-cells from G₁ phase to S phase, was also inhibited by oxLDLs whereas expression of other activation antigens such as CD69 and HLA-DR was unchanged. In conclusion, these data show that mildly oxidized LDLs inhibit the proliferation and CD25 expression of activated T-lymphocytes and suggest that oxLDLs may slow down the T-cell response in atherosclerotic lesions.

INTRODUCTION

Low-density lipoproteins (LDLs) play a major physiological role in delivering cholesterol and other lipids to peripheral cells [1]. After oxidation, they are thought to be involved in the pathogenesis of atherosclerotic lesions [2,3], and pleiotropic effects on various cellular functions have been shown. Oxidized LDLs (oxLDLs) are for example able to induce activation-related signals [4,5], to modulate expression of growth factors, adhesion molecules and tissue factors [6,7], to be mitogenic to vascular smooth-muscle cells [8] and to stimulate monocyte and T-cell migration [9,10].

LDL oxidation is a progressive process leading at first to the formation of mildly oxidized LDLs, which have a low content of lipid peroxidation derivatives and only slight apolipoprotein B (apoB) modification. Extensively oxidized LDLs contain high levels of lipid peroxidation products and show extensive apoB alteration [3,11]. LDL oxidation can be mediated by cells present in atherosclerotic lesions (endothelial cells, smooth-muscle cells, macrophages, lymphocytes) [12,13]. LDL oxidation is promoted by cell-derived free radicals and reactive oxygen species [13,14], by transition metals such as iron or copper [11], thiol compounds, H₂O₂/myeloperoxidase/HClO and tyrosyl radicals [13]. LDLs can also be oxidized by UV radiation [15,16], which is an easy way to obtain mildly oxidized LDLs, characterized by relatively low levels of peroxidation products without major apoB alter-

ation [16]. These oxLDLs may be representative of the initial steps of the oxidative attack of LDL; they are taken up through the apoB/E receptor pathway [17] and exhibit various dose-dependent biological effects [4,8,17].

T-cells are present in early atherosclerotic lesions and may constitute up to 20% of cells in the fibrous cap of advanced human lesions, but their role in atherogenesis is largely unknown [18–20]. They constitute a polyclonal population [19]. Both CD4⁺ and CD8⁺ T-cell subtypes are found in human lesions, and a recent study in cholesterol-fed rabbits indicates that the majority of T-lymphocytes in early lesions of atherosclerosis are CD4⁺ helper T-cells [18]. The presence of activation markers (e.g. CD25, HLA-DR and VLA1) and many cytokines (e.g. interferon γ, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor and tumour necrosis factor) [20–22] suggests that these T-cells are immunologically active. The mechanisms involved in activation of T-lymphocytes observed in the lesions are poorly understood. The presence of specific antigens such as oxLDLs, heat-shock proteins and viral proteins has been evoked [21,22]. Among the putative antigens, oxLDLs have attracted considerable interest recently. Indeed, T-cell clones recognizing oxLDLs in the presence of monocytes have been established from atherosclerotic plaques [23], and oxLDLs may induce humoral immunity, as shown by the presence of oxLDL antibodies in hypercholesterolaemic rabbits and in atherosclerotic patients [21,22]. OxLDLs are able to activate

Abbreviations used: LDL, low-density lipoprotein; oxLDL, oxidized LDL; apoB, apolipoprotein B; PMBC, peripheral blood mononuclear cell; IL, interleukin; LPDS, lipoprotein-depleted serum; TNBS, 2,4,6-trinitrobenzene sulphonic acid; TBARS, thiobarbituric acid-reactive substances; LysoPtdchol, lysophosphatidylcholine; FCS, fetal calf serum; PBL, peripheral blood lymphocyte; HA, haemagglutinin A; PHA, phytohaemagglutinin; mAb, monoclonal antibody.

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T-cells from peripheral blood mononuclear cells (PBMCs) of healthy donors *in vitro* through monocyte-mediated processing [12].

OxLDLs induce production of interleukin (IL)1 β and IL-12 in resting (unactivated) monocytes [12,24], but inhibit tumour necrosis factor- α and IL- β mRNA and protein expression in lipopolysaccharide-activated macrophages [25]. The effect of oxLDLs on monocytes is thus dependent on their activation state, and lymphocytes may also behave differently according to their activation state.

As oxLDLs have been shown to alter various cell functions, it was interesting to investigate their effect on human activated T-lymphocytes. We used polyclonal lymphocyte populations from peripheral blood, an immortalized CD4⁺ T-cell line and a CD4⁺ T-cell clone. The use of CD4⁺ lymphocytes is of interest since they play a central role in the initiation and development of immune responses. The present data mainly show that mildly oxidized LDLs suppress the proliferation and expression of IL-2 receptor α -chain (CD25) of activated T-lymphocytes.

MATERIALS AND METHODS

LDL isolation and oxidation

LDLs ($d = 1.019\text{--}1.063$) and lipoprotein-depleted serum (LPDS) were isolated from pooled fresh human sera by sequential ultracentrifugation as previously described [16]. LDLs were dialysed against 150 mM NaCl containing 0.3 mM EDTA, sterilized by filtration (0.2 μm Millipore membrane) and stored at 4 °C (stock solution, about 6 mg of apoB/ml) under nitrogen until use (up to 2 weeks). Under the standard conditions, LDLs were oxidized by UV radiation. ApoB solution (2 mg/ml) was exposed to UV-C radiation (254 nm; 0.5 mW/cm²) for 2–2.5 h or for various times as indicated. The oxidation state of the LDLs was estimated by determination of: (1) reactive amino groups using the 2,4,6-trinitrobenzenesulphonic acid (TNBS) method [26]; (2) thiobarbituric acid-reactive substances (TBARS) by the fluorimetric procedure of Yagi [27]; (3) the formation of lysophosphatidylcholine (LysoPtdchol) as previously described [28] (briefly, after extraction from LDLs, lipids were separated by TLC, and then phosphatidylcholine and LysoPtdchol were scraped off and their phosphorus contents determined); (4) hydroperoxide concentrations by use of a colorimetric commercial kit (PeroXOquant[®]; Pierce, Rockford, IL, U.S.A.) based on the oxidation of ferrous to ferric ion in the presence of xylenol orange [29]; (5) total oxysterols by GLC, using a chromatograph coupled to a mass spectrometer as previously reported [16]. As expected, UV-C radiation of LDLs causes only minor structural alterations of the apoB since no significant modification of TNBS-reactive amino groups was observed (Table 1). In our experimental conditions, the 2 h UV-C radiation mainly oxidizes the lipids, as shown by an increase in TBARS, hydroperoxides and total oxysterols. However, the percentage of lysoPtdchol was stable. In addition, hydroperoxides and TBARS increased similarly when LDLs were oxidized for 1, 2 and 3 h, suggesting that the UV oxidation is a dynamic process and, in agreement with our previous observations [16,17,28], that the LDL oxidation is always in its early phase, i.e. before the maximum concentrations of TBARS and hydroperoxides are attained. The presence of endotoxin in the preparations was analysed using the *Limulus* assay (QLC-1000; Boehringer Bioproducts, Heidelberg, Germany), according to the manufacturer's instructions.

Alternatively, mildly oxidized LDLs were obtained by incubating LDLs with subconfluent human endothelial cells (CRL-1998; ATCC, Rockville, MD, U.S.A.). Cells were grown for 24 h in RPMI 1640 (Gibco, Cergy Pontoise, France) supplemented

with 10% fetal calf serum (FCS) (Gibco), then grown in serum-free RPMI 1640 for 12 h and finally incubated for 18 h with LDLs (100 μg of apoB/ml) in serum-free RPMI 1640. Under these conditions, cell-derived oxLDLs contained 1.1 ± 0.3 nmol of TBARS/mg of apoB and 84 ± 10 nmol of hydroperoxides/mg of apoB.

Cell culture and activation of T-cells

Cell lines were cultured in RPMI 1640 supplemented with glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), sodium pyruvate (1 mM) and 10% FCS. During experiments, the FCS was replaced by 2% LPDS.

Experiments were carried out on three different lymphocyte populations. (1) PBMCs were separated by centrifugation (20 min; 1500 g) over Ficoll (Gibco) from heparinized venous blood of healthy donors (five different donors were used). Interface cells were washed twice in RPMI 1640. Peripheral blood lymphocytes (PBLs) were obtained by removing adherent cells after PBMC incubation for 2 h at 37 °C in 60 mm plastic Petri dishes. The PBL suspensions contained approx. 75–85% T-cells, 5–10% B-cells, 10–15% natural killer cells and 0.5–1% monocytes. (2) A transformed human T-cell line, BR/B, was obtained after stimulation of PBMCs from a healthy donor (BR) by an irradiated allogeneic B-cell line (BB) (HLA-A1, 3; B35, 44; Cw4, DR103, DQ1). Five cycles of stimulation were used, then the cells were immortalized by infection with Herpes virus saimiri as described previously [30,31]. The cell line was CD3⁺ and CD4⁺ (99% and 98% of positive cells) and CD8[−] and CD56[−], with a Th1 type (IL-2⁺, IFN γ ⁺ and IL-4[−]). (3) A human CD4⁺ T-cell clone W1833 (Th1 type), specific for influenza virus haemagglutinin A (HA) peptide (306–318; Neosystem Laboratory, Strasbourg, France) and restricted by HLA-DR1 as previously described [32] was also used.

Cells were activated as follows, in the presence (or not) of LDLs or oxLDLs (concentrations expressed as μg of apoB/ml). PBLs were activated by phytohaemagglutinin (PHA), a known polyclonal T-cell activator (PHA-P; 1 $\mu\text{g}/\text{ml}$; Sigma-Merck, Darmstadt, Germany). Immortalized CD4⁺ T-lymphocytes (BR/B) were activated by irradiated allogeneic lymphoblastoid B cells (BB). The W1833 CD4⁺ T-cell clone was activated specifically by HA peptide presented by the irradiated autologous lymphoblastoid B cell line (JPC) (HLA-A2, 3; B44, 55; DR1, DQ1).

Proliferative assays and cell-viability evaluation

The toxic effect of oxLDLs on PHA-activated lymphocytes was monitored by two tests: (i) detection of necrotic cells (i.e. cells with plasma-membrane alteration) by Trypan Blue staining; (ii) detection of apoptotic cells by flow cytometry using anti-Apo2.7 monoclonal antibody (mAb) which identifies a 38 kDa antigen associated with the mitochondrial membrane and preferentially expressed at the surface of cells undergoing apoptosis [33]. This antigen can be identified after apoptosis induction via CD95, irradiation or drug treatment. Cell viability of other activated lymphocytes was evaluated exclusively by Trypan Blue exclusion.

Proliferation was measured by the standard [³H]thymidine-uptake assay as previously described [32]. Briefly, cells ($10^5/\text{ml}$, in RPMI 1640 supplemented with 2% LPDS) were seeded in 96-well plates (100 $\mu\text{l}/\text{well}$). The cells were incubated with different reagents from 24 to 72 h at 37 °C, and 1 μCi of [³H]thymidine (1 Ci = 37 GBq; ICN, Orsay, France) was added to each well for the last 16 h. Cells were harvested on glass-fibre filters by means of a semi-automatic cell harvester (Filtermate 196; Packard, Rungis, France), and the amount of incorporated [³H]thymidine

Table 1 Comparison of oxidation parameters of native LDLs and LDLs mildly oxidized using UV light

LysoPtdchol formation is expressed as a percentage of lysoPtdchol/lysoPtdchol + Ptdchol. Results are means $\pm$ S.E.M. from four to nine experiments.

	Native LDLs	Mildly oxidized LDLs
TNBS (% of control)	100 $\pm$ 4	95 $\pm$ 3
TBARS (nmol/mg of apoB)	0.3 $\pm$ 0.1	4.3 $\pm$ 0.6
Hydroperoxides (nmol/mg of apoB)	12.5 $\pm$ 2.3	75.6 $\pm$ 8.1
LysoPtdchol (%)	3 $\pm$ 1	4 $\pm$ 2
Total oxysterols (μ g/mg of apoB)	0.7 $\pm$ 0.2	1.5 $\pm$ 0.3

was determined on a Matrix 9600 counter (Packard). The mean radioactivity (c.p.m.) from triplicate cultures was used for calculations.

Flow cytofluorimetric analysis

Tricolor-conjugated anti-CD25 (α -chain of IL-2 receptor) and phycoerythrin-conjugated anti-CD4 (helper T-cell marker) mAbs were purchased from TEBU (Le Perray en Yvelines, France). FITC-conjugated anti-CD69 mAb (CD69 is among the early markers that appear after the activation of lymphocytes) was obtained from Becton Dickinson (Mountain View, CA, U.S.A.). Phycoerythrin- or FITC-conjugated anti-CD3 (specific T-cell marker) mAbs, phycoerythrin-conjugated anti-Apo2.7 (early apoptosis cell marker) and anti-HLA-DR (HLA-DR is among the late markers that appear after the activation of T-lymphocytes) mAbs were purchased from Immunotech (Marseille, France). FITC-PHA was supplied by Sigma. Staining of lymphocytes was performed as previously described [34]. Ten thousand events were collected, stored and analysed on a Coulter Elite cytofluorimeter.

For the studies of membrane antigen expression, forward-angle and 90° light-scatter gates were established to exclude cell debris from analysis, then the lymphocyte population was selected using cell size and cell granularity criteria. Two fluorescence parameters were used: (i) the percentage of cells exhibiting fluorescence that exceeds the upper limit of autofluorescence of cells incubated with an irrelevant mAb (positive cells); (ii) the mean fluorescence intensity of the positive cells in arbitrary units.

Cell-cycle studies were carried out by the method of Vindeløv and Christensen [35]. The fluorescence intensity from cell nuclei stained with propidium iodide is proportional to the cellular DNA content. Cell cycle was analysed using the Multicycle Phoenix Flow System (P. F. Rabinovitch, University of Washington, Seattle, WA, U.S.A.).

RESULTS

Effect of oxLDLs on viability of PHA-activated lymphocytes

Under the experimental conditions used here, up to 200 μ g/ml oxLDLs had only a minor effect on the viability of activated lymphocytes, since no significant difference was seen in Trypan Blue uptake over the 72 h of the experiment (Figure 1A). The effect of oxLDLs on induction of apoptosis of activated lymphocytes was studied using cytofluorimetry and the Apo2.7 mAb [33]. The percentage of Apo2.7-positive cells was evaluated in the lymphocyte population gated by cell size and cell structure criteria. After 3 days of culture, 200 μ g/ml oxLDLs induced a slight increase in the percentage of Apo2.7-positive cells, whereas

Figure 1 Effect of oxLDLs on viability of PHA-activated lymphocytes

PHA-activated PBLs were incubated in RPMI/2% LPDS with different concentrations of native LDLs (□) or oxLDLs (◆) (TBARS 3.5 ± 0.2 nmol/mg of apoB) for 72 h (A and B; for clarity, results obtained with 1, 2.5 and 5 μ g/ml are not shown). In the insets are shown the values obtained with 200 μ g/ml at three different time points. +, Control values (without LDLs added to the cells). The percentage of necrotic cells stained by Trypan Blue was determined using a light microscope (A), and the percentage of apoptotic cells was determined by detection of Apo2.7 protein using a cytofluorimeter (B). Values are means from triplicate determinations. Two other separate experiments, each using a different donor, gave identical results.

no significant increase in Apo2.7-positive lymphocytes was observed up to 100 μ g/ml oxLDL (Figure 1B).

Effect of oxLDLs on proliferative response of PHA-activated lymphocytes

To study the potential alteration of T-cell proliferation by oxLDLs, PBLs were incubated simultaneously with PHA and LDLs or oxLDLs. After 3 days of culture, DNA synthesis was measured by [3 H]thymidine uptake. Native LDLs increased the proliferation at concentrations of 10 to 100 μ g/ml and inhibited at 200 μ g/ml, whereas oxLDLs inhibited the proliferation at all these doses (Figure 2A). For clarity, results with 1, 2.5 and 5 μ g/ml LDL and oxLDL are not shown on Figure 2(A), but no significant effect was observed on proliferation. When the results were normalized as a percentage of the response

Figure 2 Effect of oxLDLs on proliferation of PHA-activated lymphocytes

Cell proliferation was estimated by determination of [³H]thymidine uptake. (A) PHA-activated PBLs were incubated with different concentrations of native LDLs (□) or oxLDLs (◆) (TBARS 4.1 ± 0.3 nmol/mg of apoB) for 72 h in RPMI/2% LPDS, and cell viability was checked using the Trypan Blue-exclusion test (inset). Results are expressed as means from triplicate cultures and are representative of four independent experiments performed with PBLs from different donors. (B) The results from (A) and from three other experiments are expressed as percentage of radioactivity (CPM) observed after 3 days: PHA-activated PBLs in the presence of LDLs (□) or oxLDLs (◆). Results are means $\pm$ S.D. * $P < 0.05$, compared with LDL-treated cells for the same concentration of lipoproteins; ** $P < 0.02$, compared with cells treated with 10 μ g/ml LDL (Student's *t* test). (C) PHA-activated PBLs were incubated with 0, 10, 100 and 200 μ g/ml oxLDLs (TBARS 3.3 ± 0.2 nmol/mg of apoB) for 24, 48 and 72 h. A second experiment gave similar results. (D) PHA-activated PBLs were cultured for 72 h with 100 μ g/ml native LDLs, with LDL-free culture supernatant of human endothelial cells (HEC SN) without LDLs or with LDLs added at 0 h of culture, and with LDLs oxidized for 18 h by human endothelial cells (HEC-oxLDL, TBARS 1.1 ± 0.3 nmol/mg of apoB; hydroperoxides 84 ± 10 nmol/mg of apoB). * $P < 0.05$, compared with cells cultured in the presence of LDLs or with HEC SN + LDLs (Student's *t* test). Results are means $\pm$ S.D. from three independent experiments performed with lymphocytes from the same donor. Two other experiments performed with lymphocytes from two other donors showed identical results.

of PHA-activated PBLs without LDLs, the inhibitory effect of 200 μ g/ml LDL and 50–200 μ g/ml oxLDL is clearly seen (Figure 2B). The inhibition induced by oxLDLs is analysed further in Figure 2(C) showing an effect of both dose and time. The inhibitory effect of oxLDLs cannot be attributed to cell death, since no significant toxicity was observed up to 100 μ g/ml oxLDL (Figures 1 and 2A). In addition, it is unlikely that this effect was due to a direct effect of oxLDLs on PHA (or on the CD3–T-cell receptor complex), since oxLDLs did not inhibit the binding (at 4 °C and 37 °C) of FITC–PHA (and phycoerythrin–anti-CD3) to lymphocytes (using flow cytofluorimetric analysis; results not shown).

To study the effect of LDLs oxidized by a different method, LDLs were oxidized by human endothelial cells. Human endothelial-cell supernatant alone appeared to stimulate cell proliferation as compared with control cells cultured with 100 μ g/ml LDL (Figure 2D). The addition of 100 μ g/ml LDL to the endothelial-cell supernatant inhibited [³H]thymidine uptake in activated PBLs compared with that in PBLs cultured in human endothelial cell supernatant alone. This LDL-inhibitory effect is similar to that observed in Figure 2(B). The addition of LDLs oxidized by human endothelial cells significantly increased the

inhibitory effect on cell proliferation (Figure 2D). The effect of LDLs oxidized in this way is thus similar to that observed with LDLs mildly oxidized by UV, although the level of lipid peroxidation was slightly different (after 18 h culture, the TBARS concentrations were lower and the hydroperoxide concentrations barely higher). We preferred to use LDLs oxidized by UV in subsequent experiments, since preparations of cell-oxidized LDLs may contain enzymes, growth factors or cytokines able to interfere with PBL proliferation. Finally, the experiments with cell-oxidized LDLs agree with the conclusion that mild oxidation of LDLs, characterized by moderate levels of TBARS and hydroperoxides, may induce inhibition of T-cell proliferation.

To clarify the relationship between the level of lipid peroxidation of LDLs and the inhibitory effect on PBL proliferation, experiments were carried out with LDLs oxidized by UV irradiation for various times (1, 2 and 3 h). As shown in Figure 3, the inhibition correlated directly with the irradiation time and the TBARS level (and the hydroperoxide level; not shown) at day 0 of culture.

We have examined further the possibility that activated PBLs may oxidize LDLs or increase the oxidation level of oxLDLs during cell culture. In our experimental conditions activated

Figure 3 Relationship of LDL oxidation state to inhibition of [^3H]thymidine incorporation in PHA-activated lymphocytes

LDLs were oxidized for 1, 2 or 3 h by UV irradiation. PHA-activated PBLs were incubated for 72 h with 0, 10, 100 and 200 $\mu\text{g/ml}$ native LDLs ($\square$) or oxLDLs ($\blacklozenge$), and cell proliferation was estimated by determination of [^3H]thymidine uptake. The inset (simple regression) shows that at 100 $\mu\text{g/ml}$ oxLDL, the inhibition is proportional to the TBARS level. Results are expressed as means from triplicate determinations and are representative of two independent experiments, each performed with a different donor.

Figure 4 Effect of oxLDLs on [^3H]thymidine incorporation in activated CD4^+ T-lymphocytes

CD4^+ T-cell line, BR/B, activated by allogenic cells (A), CD4^+ T-cell clone W1833 activated by HA peptide and autologous cells (B) were incubated with various concentrations of native LDLs ($\square$) or oxLDLs ($\blacklozenge$) for 72 h in RPMI/2% LPDS (for clarity, results obtained with 1, 2.5 and 5 $\mu\text{g/ml}$ are not shown). Results are expressed as means $\pm$ S.D. from three independent experiments performed in triplicate. $^*P < 0.05$, compared with DNA synthesis in cells cultured in the absence of lipoprotein (Student's t test).

lymphocytes have insignificant oxidative power on LDLs (not shown).

Effect of oxLDLs on activated CD4^+ T-lymphocytes

The T-cell population is heterogeneous with respect to both functional capabilities and cell-surface phenotype. Broadly speaking, T-cells are divided into CD4^+ and CD8^+ lymphocytes. The CD4^+ helper T-cells have a central role in the development of specific immune responses by providing signals that are

Table 2 Effect of oxLDLs on the cell cycle of a CD4^+ T-cell clone

The CD4^+ T-cell clone W1833 activated specifically was incubated with LDLs or oxLDLs at 100 $\mu\text{g/ml}$ and 300 $\mu\text{g/ml}$ for 72 h in RPMI/2% LPDS. The cell cycle was analysed by cytofluorimetry by the method of Vindeløv and Christensen [35]. Results are expressed as percentage of cells in G_0/G_1 , S and G_2/M phases and are means $\pm$ S.D. from three independent experiments.

	G_0/G_1 phase	S phase	G_2/M phase
Control cells	64 $\pm$ 3	25 $\pm$ 3	11 $\pm$ 2
LDL (100 $\mu\text{g/ml}$)	63 $\pm$ 3	27 $\pm$ 4	10 $\pm$ 3
LDL (300 $\mu\text{g/ml}$)	65 $\pm$ 3	25 $\pm$ 3	10 $\pm$ 3
oxLDL (100 $\mu\text{g/ml}$)	76 $\pm$ 1	18 $\pm$ 3	6 $\pm$ 2
oxLDL (300 $\mu\text{g/ml}$)	96 $\pm$ 2	2 $\pm$ 1	1 $\pm$ 1

Figure 5 Time-dependent effect of oxLDLs on the expression of CD25 at the cell surface of PHA-activated PBLs

PHA-activated PBLs were incubated in RPMI/2% LPDS without (+) or with 100 $\mu\text{g/ml}$ native LDLs ($\square$) or oxLDLs ($\blacklozenge$) for 24, 48 and 72 h. After staining with anti-CD25 mAb, cell suspensions were studied using a cytofluorimeter. CD25 expression was studied in the total lymphocyte population, selected by cell size and cell granularity criteria, and excluding cell debris and residual monocytes (A and B), in Apo2.7-negative lymphocytes (non-apoptotic) (C and D) or in CD3^+ lymphocytes (T-lymphocytes) (E and F). Results are expressed as means of CD25^+ cell percentage (A, C and E) and of mean fluorescence intensity (B, D and F) from triplicate determinations, and are representative of three independent experiments, each performed with a different donor.

necessary for B-cells and cytotoxic cells to differentiate into effector cells. To investigate the effect of oxLDLs on activated CD4^+ T-lymphocytes, the proliferative response of an oligoclonal CD4^+ T-lymphocyte cell line and a CD4^+ T-cell clone was

Figure 6 Dose-dependent effect of oxLDLs on the expression of CD25 in activated CD3⁺ or CD4⁺ T-lymphocytes

PHA-activated PBLs (**A**) and CD4⁺ T-cell clone W1833 activated by HA peptide (**B**) were incubated in RPMI/2% LPDS with 0, 10, 50, 100 and 200 µg/ml native LDLs (□) or oxLDLs (◆) for 72 h, and the expression of CD25 was then studied in the CD3⁺ lymphocyte population and in the CD4⁺ population. Results are expressed as CD25 mean fluorescence intensity in double-positive populations (CD3⁺CD25⁺ or CD4⁺CD25⁺) and are expressed as means from triplicate determinations. They are representative of two independent experiments.

studied in cultures containing oxLDLs. The CD4⁺ T-lymphocyte cell line BR/B was activated by incubation with allogenic cells, and the CD4⁺ T-cell clone W1833 by its specific HA peptide presented by DR1⁺ cells. After 72 h of culture, DNA synthesis was measured by [³H]thymidine uptake (Figure 4). As previously observed, LDLs enhanced the proliferation at low doses, but the effect decreased with higher doses, 200 µg/ml tending to be inhibitory. OxLDLs inhibited DNA synthesis in a dose-dependent manner in both the BR/B cell line and the W1833 clone. These observations suggested that oxLDLs act directly on activated T-lymphocytes.

To investigate a possible block of the cell cycle, the CD4⁺ T-cell clone W1833 was specifically activated as previously described in the presence of 100 and 300 µg/ml LDL or oxLDL. Cell nuclei were analysed by cytofluorimetry at 72 h for staining with propidium iodide (Table 2). With 100 µg/ml oxLDL, the number of W1833 cells in G₁ phase increased significantly (from 64 ± 3% in control cells to 76 ± 1%; means of three experiments; $P < 0.01$) with a corresponding decrease in cells in S and G₂/M phases (from 25 ± 3 to 18 ± 3% and 11 ± 2 to 6 ± 2% respectively; $P < 0.05$) whereas 100 µg/ml LDL had no significant effect (G₁ = 63 ± 3%; S = 27 ± 4%; G₂/M = 10 ± 3%). Similar results were observed with PHA-activated PBLs (not shown). With 300 µg/ml oxLDL, the block in G₁ phase was very clear (96 ± 2%), the number of cells in S and G₂/M phases decreasing strongly (S = 2 ± 1% and G₂/M = 1 ± 1%). These results suggest that oxLDLs induced a block at the G₁/S interface, involving a decrease in cells in the S and G₂/M phases. In addition, the DNA profile in cells incubated with the highest concentration of oxLDLs showed DNA fragmentation seen as a peak with lower fluorescence than DNA in G₁ phase (as a relative percentage of total cells: 6 ± 2% in control cells versus 6 ± 2% in 100 µg/ml oxLDL-treated cells and 25 ± 3% in 300 µg/ml oxLDL-treated cells), indicating cell death.

Effect of oxLDLs on the expression of CD25

Most lymphocytes in peripheral blood are in a resting state (G₀) and are CD25⁻. Stimulation of T-lymphocytes with PHA or a

Figure 7 Comparative effect of oxLDLs on expression of different membrane molecules of T-lymphocytes

PHA-activated PBLs were incubated for 72 h in RPMI/2% LPDS with 0, 10, 100 and 200 µg/ml oxLDLs (**A**), or with 100 µg/ml LDL oxidized by UV for 1, 2 and 3 h (**B**). Expression of CD25 (□), CD3 (+), CD4 (×), CD69 (◆) and HLA-DR (★) was studied in the total lymphocyte population using cytofluorimetric analysis. Results are expressed as means of triplicate determinations and are representative of two independent experiments.

specific peptide induces the transition from G₀ to G₁ and concomitantly the expression of CD25 (α-chain of IL-2 receptor) which associates non-covalently with β- and γ-subunits to form the high-affinity IL-2 receptor. For the study of the effect of oxLDLs on the expression of CD25 in PHA-activated PBLs, we used two parameters of cytofluorimetry: the percentage of CD25⁺ cells and the mean fluorescence intensity of positive cells, which is dependent on the number of CD25 molecules/cell. The presence of oxLDLs (100 µg/ml) during the proliferative response of PHA-activated PBLs decreased the level of CD25 expression in the total lymphocyte population (natural killer, and T-lymphocytes) (Figures 5A and 5B) and in the T-lymphocyte population defined as CD3⁺ cells (Figures 5E and 5F). To exclude the possibility that oxLDLs might react with the critical epitope of the mAb used, CD25 staining (30 min; 4 °C) was performed in the presence of oxLDLs (200 µg/ml) on PHA-activated PBLs cultured for 72 h without LDLs. This did not change the percentage of CD3⁺CD25⁺ lymphocytes (not shown). In addition the inhibition of CD25 expression seemed to be independent of apoptosis (Figures 5C and 5D). The inhibition was clearly dose-dependent (Figure 6A) and also observed in the CD4⁺ T-cell clone W1833 specifically activated by HA peptide (Figure 6B).

We further examined whether the inhibitory effect of oxLDLs was specific for CD25 or affected more generally the expression of other membrane molecules (CD3, CD4, CD69 and HLA-DR). Cytofluorimetric study of PHA-activated PBLs during 72 h of co-culture with increasing concentrations (10, 100, 200 µg/ml) of oxLDLs (Figure 7A) showed no change in

expression in contrast with CD25 expression. This result was confirmed by experiments using LDLs with variable oxidation levels. The CD25 expression decreased with an increase in TBARS level (and hydroperoxide level; not shown), whereas CD3 and CD69 expression was not inhibited (Figure 7B).

In conclusion, oxLDLs inhibit the expression of CD25 but not the expression of other activation molecules such as CD69 and HLA-DR. The constitutive expression of CD3 or CD4 on T-cells was not changed.

DISCUSSION

The immunoregulatory effect of LDLs has been demonstrated previously in PHA-activated PBLs [36]. The present study shows that low concentrations of native LDLs enhance DNA synthesis in activated T-lymphocytes whereas higher concentrations inhibit the proliferative response, independently of the complexity of T-lymphocyte populations (polyclonal CD3⁺ cells in PBLs, oligoclonal and monoclonal CD4⁺ lymphocytes). Indeed, the modulation of DNA synthesis was reproducibly observed in well-defined activated T-cells such as a CD4⁺ T-cell clone, suggesting a direct effect of LDLs on T-cells. The stimulating effect of LDLs on T-cell proliferation may be due to the cholesterol requirement for cell proliferation and/or to a general cellular activation through stimulation of a signalling pathway [36,37]. Recently, it has been demonstrated that LDLs induced the expression of the early growth response gene-1 (*egr-1*) [38]. On the other hand, the previously reported [36,37] inhibitory effect of high LDL concentrations, remains surprising. It is tempting to speculate on the involvement of the putative immunoregulatory LDL receptor with low affinity, hypothesized by McCarthy et al. [37], which would be able to mediate inhibition of human activated lymphocytes.

The present data indicate that the T-lymphocyte response can be inhibited by oxLDLs (decrease in DNA synthesis, inhibition of CD25 expression and blockage of cell cycle) independently of the type of T-cell activator (PHA, allogenic cells, specific peptide + antigen-presenting cells) and of the complexity of the T-cell population. In addition, the inhibitory effect on cell proliferation and CD25 expression is time- and dose-dependent. This is consistent with the assumption that oxLDLs must be taken up by cells and must reach a minimal threshold before triggering an effect, as previously reported in several experiments [4,7,8,10].

OxLDLs inhibit CD25 expression at the cell surface, whereas the expression of CD3 and CD4 (constitutively expressed on T-cells), CD69 (early activation antigen) and HLA-DR (late activation antigen) was unchanged. Thus oxLDLs do not alter the steps of the signalling pathways triggering CD69 and HLA-DR expression, but mild oxidation of LDLs was sufficient to dramatically disturb CD25 expression. The inhibition of CD25 expression induced by oxLDL correlated with decreased DNA synthesis and cell cycle arrest at the G₁/S interface. Activation of T-lymphocytes is known to induce the commitment of cells from G₀ to G₁, the concomitant expression of CD69 and CD25 and IL-2 synthesis [39]. The transition from G₁ to S depends on the interaction of IL-2 with the IL-2 receptor. Consequently, it can be proposed that the inhibition of CD25 expression is involved in the blocking of cells in the G₁ phase and the inhibition of DNA replication. However, it is impossible to distinguish between G₀ and G₁ phases using cytofluorimetry, and the possibility of an exodus of cells from the cell cycle towards the G₀ state cannot be ruled out.

On the other hand, the inhibitory effect correlates with the level of TBARS in the medium at the time of activation. In

agreement with a report showing that lipid peroxides of oxLDLs may be able to inhibit the migration of endothelial cells [40], we propose that lipid peroxidation products may contain inhibitors of T-lymphocyte response since LDLs mildly oxidized by UV are characterized by preferential lipid oxidation [16,17]. Since oxLDLs can elicit various intracellular transduction signals [4,5], the inhibition could be due to an alteration in the homeostatic regulation of normal intracellular signals of T-cell response. Another possibility is that oxLDLs increase the concentration of reactive oxygen intermediates leading to an oxidative shift in the cellular redox state, which modifies the nature of the stimulatory signal and results in cell-cycle arrest [41].

OxLDLs are able to modulate functions of cells present in the vessel wall. For example, oxLDLs are chemotactic for monocytes [9] and stimulate monocyte adhesion to endothelial cells [6] and the production of some colony-stimulating and tissue factors, e.g. IL-1 β and IL-12 in unactivated monocytes [12,24]. On the other hand, oxLDLs can inhibit lymphocyte-mediated cellular cytotoxicity [42], pinocytic activity [43], migration of endothelial cells [40] and tumour necrosis factor α and IL-1 β production in activated macrophages [25]. According to the cell type and/or the activation state of the cells, and perhaps the nature of the oxidized molecules of LDL, oxLDLs may activate or inhibit cellular functions. Several groups have presented data showing that oxLDLs induce lymphocyte reactivity. Thus extensively oxidized LDLs, at concentrations between 0.5 and 10 μ g/ml, induce activation of T-cells (from peripheral blood [12] and plaques [23]) in the presence of monocytes. In these experiments, LDLs were oxidized by CuSO₄, had ≥ 20 nmol of TBARS/mg of protein and exhibited an increased rate of degradation by macrophages [12]. The present data demonstrate that mildly oxidized LDLs, characterized by 3–5 nmol of TBARS/mg of apoB, 60–90 nmol of hydroperoxides/mg of apoB and only minor structural and functional modifications of apoB [16], can strongly inhibit T-cell proliferation at concentrations higher than 10 μ g/ml. It is conceivable that low concentrations of apoB-modified LDLs in the presence of antigen-presenting cells (e.g. monocytes/macrophages) allow the specific activation of some T-cells in atherosclerotic plaques or lymph nodes. The specific T-cell response to oxLDLs and other antigens may afterwards be directly inhibited by moderate concentrations of oxLDLs. Mildly oxidized LDLs may be particularly inhibitory (compared with apoB-modified LDLs) since they can be taken up efficiently by the activated lymphocytes using the apoB/E receptor.

Although T-lymphocytes are present in atheroma, the exact role of the immune system in the formation of the lesion is not known. Recent data have provided evidence that atherosclerosis may be an immunologically mediated disease [20–22]. However, it is conceivable that lymphocytes do not always have deleterious effects, e.g. during the progression of atherosclerosis. For example, it has been recently demonstrated in rabbits that the immune response against oxLDLs has a protective effect against the development of early atherosclerotic lesions [44]. In fact, several data obtained *in vivo* suggest that immunodeficiency may facilitate the development of atherogenesis [45–47]. Our report suggests that oxLDLs can be directly immunosuppressive to the T-lymphocyte response. The hypothesis of an *in vivo* immunosuppressive effect of oxLDLs remains to be investigated.

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