Mildly oxidized low-density lipoproteins decrease early production of interleukin 2 and nuclear factor κ B binding to DNA in activated T-lymphocytes

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Activated T-lymphocytes are found early in atherosclerosis lesions, but little is known about their role. Oxidized low-density lipoproteins (oxLDLs) are considered to be involved in the pathogenesis of the lesions, and we have previously demonstrated that oxLDLs inhibit not only interleukin (IL)-2-receptor expression on the surface of *in vitro*-activated T-lymphocytes but also their proliferation. We have now investigated the effect of oxLDLs on blast differentiation, on IL-2 synthesis and on the activation of the nuclear factor κ B (NF- κ B) system in activated lymphocytes. Mildly oxLDLs (50 and 100 μ g/ml) decreased the number of lymphoblasts and the level of IL-2 concentration in the culture supernatants after activation of lymphocytes by phytohaemagglutinin and PMA + ionomycin. The inhibition of

IL-2 production was observed in the CD3⁺ T-lymphocyte cytoplasm as early as 4 h after activation by PMA + ionomycin. The study of NF- κ B showed that oxLDLs led to a decrease of activation-induced p65/p50 NF- κ B heterodimer binding to DNA, whereas the presence of the constitutive nuclear form of p50 dimer was unchanged. This was correlated with an unchanged level of the active form of the cytosolic inhibitor protein I κ B- α . Taken together, these observations suggest that the immunosuppressive effect of oxLDLs might operate via a dysregulation of the T-lymphocyte activation mechanisms.

Key words: atherosclerosis, lipoproteins, lymphokines, I κ B, immunosuppression

INTRODUCTION

Low-density lipoproteins (LDLs) deliver cholesterol and other lipids to peripheral cells [1]. Oxidized LDLs (oxLDLs) participate in the development of atherosclerotic lesions [2]. Among the pleiotropic effects on cellular functions, oxLDLs induce the production of interleukins (ILs) such as IL-1- β , IL-8, IL-10 and IL-12 in resting monocytes [3–5] and inhibit tumour necrosis factor α and IL-1 β mRNA and protein expression in lipopolysaccharide-activated macrophages [6]. oxLDLs may also activate resting lymphocytes through a monocyte-mediated process [4,7], but they inhibit the proliferation of activated peripheral-blood mononuclear cells (PBMC) and T CD4⁺ lymphocytes [8,9].

The role of lymphocytes in atherosclerosis has not been clearly defined. While some data suggest that the immune system plays only a minor role in atherogenesis [10,11], others demonstrate that T-lymphocytes participate in the progression of the lesions [12–15]. For example, atherosclerotic lesions are reduced in T-cell-deficient hyperlipidaemic mice [12]. In contrast, some authors have shown that lymphocytes could, at least in the early phase, slow down atherogenesis [16–20], e.g. the B-lymphocyte response against oxLDLs has a protective effect against lesion development in rabbits [20]. In humans, activated T-cells are present early in atherosclerotic lesions [21–23]. The mechanisms involved in T-lymphocyte activation are still unknown, but the role of specific antigens such as oxLDLs, heat-shock proteins and viral proteins has been suggested [7,22].

Activation of T-cells with antigens, phorbol esters or mitogens can trigger a programme of gene expression inducing cell

proliferation and differentiation. Among the early events induced after activation is the expression of the genes for IL-2 and CD25 (α -chain of IL-2 receptor). The secretion of IL-2 and the expression of CD25 by T-lymphocytes are necessary for their lymphoblast transformation and proliferation. Among the most important events controlling gene activation are those involving the nuclear factor κ B (NF- κ B) family [24,25]. NF- κ B is present in an inactive form in the cytoplasm of resting T-cells, bound to the inhibitor proteins termed I κ B (inhibitor of κ B). A member of this family, I κ B- α , binds the p50/p65 heterodimer of NF- κ B in the cytoplasm. After activation [e.g. by phytohaemagglutinin (PHA) or PMA + ionomycin], I κ B- α is phosphorylated, ubiquitinated and degraded, whereas the p50/p65 heterodimer translocates into the nucleus, specifically binds the DNA- κ B region and activates gene transcription [24,26]. Functional κ B elements have been found on the promoters of the IL-2 and CD25 genes [24].

In a recent work we have demonstrated that mildly oxLDLs suppress the T-lymphocyte proliferative response. This observation suggests that oxLDLs may inhibit T-cell responses in atherosclerotic lesions [9]. Mildly oxLDLs were obtained using UV radiation and were characterized by relatively low levels of peroxidation products without major apolipoprotein B (apoB) alteration, which permitted uptake of oxLDLs through the normal apoB/E-receptor pathway [27]. In the present work we have studied the effect of oxLDLs on NF- κ B activation and IL-2 synthesis, which are early events induced by activation of T-lymphocytes.

Abbreviations used: LDL, low-density lipoprotein; oxidized LDL, oxLDL; apoB, apolipoprotein B; PBMC, peripheral-blood mononuclear cell; PBL, peripheral-blood lymphocytes; IL, interleukin; LPDS, lipoprotein-depleted serum; TBARS, thiobarbituric acid-reactive substances; FCS, fetal-calf serum; PHA, phytohaemagglutinin; EMSA, electrophoretic-mobility-shift assay; LPS, lipopolysaccharide.

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MATERIALS AND METHODS

Materials

Nonidet P40 (Igepal), phytohaemagglutinin-protein, PMA, ionomycin, PMSF, leupeptin, aprotinin, pepstatin, BSA, poly(dI-dC) and saponin were supplied from Sigma (Sigma-Merck, Darmstadt, Germany). RPMI 1640 medium containing Glutamax I, penicillin, streptomycin, sodium pyruvate were purchased from Gibco (Cergy-Pontoise, France). T_4 polynucleotide kinase, NF- κ B site of the κ -light-chain enhancer (5'-AGTTGAGGGGAC-TTCCCCAGGC-3') and AP1 site (5'-CGCTTGATGAGTCA-GCCGGAA-3') were provided by Promega (Madison, WI, U.S.A.). [γ - 32 P]ATP and [3 H]thymidine were from ICN (Orsay, France). Acrylamide/bisacrylamide stock solution (40%) and lymphocyte separation medium were obtained from Eurobio (Les Ulis, France).

LDL isolation and oxidation

LDLs ($d = 1.019$ – 1.063) and lipoprotein-depleted serum (LPDS) were isolated from pooled fresh human sera, dialysed, sterilized and stored at 4 °C as previously described [27].

Mildly oxLDLs (containing mainly lipid peroxidation derivatives with only minor alterations of apoB) obtained by irradiation with UV light (UV-C, 254 nm, 0.5 mW/cm², for 2 h) [27] were used. LDLs contained 0.2 ± 0.1 nmol of thiobarbituric acid-reactive substances (TBARS)/mg of apoB and 15 ± 3 nmol of hydroperoxides/mg of apoB and oxLDLs contained 4.4 ± 0.4 nmol TBARS/mg apoB and 92 ± 11 nmol hydroperoxides/mg apoB. In some experiments LDLs were oxidized by CuSO₄ (5 μ M, 2 h, 37 °C) and contained 3.8 ± 0.8 nmol of TBARS/mg of apoB and 154 ± 13 nmol of hydroperoxides/mg of apoB under our experimental conditions.

Cell culture and activation of lymphocytes

PBMC were separated from heparinized venous blood of five healthy donors by centrifugation (20 min, 1500 g), using a lymphocyte separation medium. Peripheral-blood lymphocytes (PBL) were obtained by removing adherent cells after PBMC incubation (2 h, 37 °C) in 60-mm-diameter 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.

During experiments, cells were cultured in RPMI 1640 medium containing Glutamax I, supplemented with penicillin (100 units/ml), streptomycin (100 μ g/ml), sodium pyruvate (1 mM) and 2% LPDS. Cells were activated by PHA (1 μ g/ml) or PMA (10 ng/ml) and ionomycin (100 ng/ml). PHA is known to activate only some T-cell subpopulations in PBL (mimicking the effect of antigens that activate T-cells through T-cell receptor signalling) [24,28–31], whereas PMA + ionomycin can potentially activate all lymphocyte populations [24,28]. Previous pharmacological studies showed that the activator concentrations used are sufficient to promote T-cell stimulation, including the activation of NF- κ B, the production of IL-2 and CD25 and cell proliferation [28].

Proliferative assays and cell-viability evaluation

Proliferation was measured by the standard [3 H]thymidine-uptake assay as follows. Cells (10^5 /ml of RPMI/2% LPDS) were seeded in round-bottomed 96-well plates (100 μ l/well). Cells were incubated from 24 to 72 h at 37 °C in a humidified atmosphere with 5% CO₂. A 1 μ Ci portion of [3 H]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 (Harvester 96® Tomtec; Wallac-EG&G Instruments, Evry, France), and the amount of [3 H]thymidine (c.p.m.) incorporated was determined on Micro-beta® triluX (Wallac-EG&G instruments). The mean radioactivity from triplicate cultures was used for calculation. In some experiments, recombinant IL-2 (generously given by SANOFI, Labège, France) was added. The cell viability was determined by the Trypan Blue-exclusion test and detection of apo2.7 antigen by flow-cytofluorimetric analysis.

Flow-cytofluorimetric analysis

PBL were surface-labelled with phycoerythrin-conjugated anti-CD3 (IOT, Marseille, France) and Tri Color-conjugated anti-CD25 antibodies (Tebu, Le Perray en Yvelines, France). Lymphocyte and lymphoblast events were gated on the side scatter (granularity) and forward scatter (size) dot-plot and selected cells were then analysed by double fluorescence.

Intracytoplasmic cytokine (IL-2) detection was performed according to a method used for the examination of cytokine kinetics in Th1/Th2 balance (L. Rostaing, J. Tkaczuk and M. Durand, unpublished work; full details are available from M.D. on request). Briefly, after 0.3% formaldehyde/PBS fixation, cells were first labelled at the surface with a peridinin-chlorophyll-protein-conjugated anti-CD3 antibody (Becton-Dickinson, Le Pont de Claix, France) and then perforated with 0.1% saponin/PBS. An appropriate dilution of a phycoerythrin-conjugated anti-IL-2 antibody (Becton-Dickinson) was then added for a 30 min incubation. After washing, cells were fixed in 0.5% formaldehyde/PBS before cytofluorimetric acquisition.

Preparation of cell extracts

PBL were harvested and washed twice in cold PBS, resuspended in 0.4 ml (per 10^7 cells) of buffer A (10 mM Hepes/10 mM KCl/1 mM MgCl₂/1 mM dithiothreitol/0.1 mM EGTA/0.5 mM EDTA/1 mM PMSF/2 μ g/ml leupeptin/10 μ g/ml aprotinin/2 μ g/ml pepstatin), and incubated on ice for 20 min. A 25 μ l portion of 10% Nonidet P40 was next added, cells were mixed vigorously for 10 s and centrifuged (1200 g, 20 min). Supernatants corresponding to the cytosolic fraction were used directly for immunoblotting. Pelleted nuclei were washed once with buffer A, resuspended in 40 μ l of buffer C (20 mM Hepes/400 mM NaCl/1 mM MgCl₂/1 mM dithiothreitol/0.1 mM EGTA/0.5 mM EDTA/1 mM PMSF/2 μ g/ml leupeptin/10 μ g/ml aprotinin/2 μ g/ml pepstatin), incubated on ice for 30 min and centrifuged (20 000 g, 20 min). The supernatants containing the nuclear proteins were used for electrophoretic-mobility-shift assay (EMSA) analyses. Protein concentration was measured with a commercial kit (Bio-Rad protein assay, Bio-Rad Laboratories, München, Germany).

Immunoblotting analysis

After heating, 60 μ g of cell protein was resolved by SDS/PAGE on 10%-acrylamide gels and transferred on to nitrocellulose membrane (Hybond-C; Amersham International, Amersham, Bucks., U.K.). The specific antigens were detected with anti-I κ B- α and anti- β actin antibodies (Tebu) and revealed by the Amersham enhanced-chemiluminescence (ECL®) method.

EMSA

The double-strand NF- κ B oligonucleotide was end-labelled using [γ - 32 P]ATP and T_4 polynucleotide kinase and purified on a commercial column (Qiaquick; Qiagen, Hilden, Germany). Reactions with equal amounts of nuclear extracts (2.5 μ g/reaction)

were carried out in 20 μ l of buffer [2 mM Hepes/50 mM NaCl/1 mM $MgCl_2$ /2.5 mM dithiothreitol/0.5 mM EDTA/4 % glycerol/200 μ g/ml BSA/6 μ g/ml poly(dI-dC) containing a probe with 100000 c.p.m., for 30 min at room temperature. Samples were loaded on a native 6%-polyacrylamide gel containing 0.5 $\times$ TBE (50 mM of Tris/borate/2 mM EDTA, pH 8). After electrophoresis, dried gels were exposed at $-80^\circ C$. Competition assays were carried out with 1 μ g of specific anti-p50 or anti-p65 antibodies (Tebu) and a 100-fold molar excess of unlabelled oligonucleotides.

Enzyme immunoassay

Cells were activated as described above. Extracellular secreted IL-2 was detected with a commercial kit (Immunotech, Marseille) according to the supplier's recommendations.

RESULTS

Effect of oxLDLs on DNA synthesis and lymphoblast recruitment after PHA activation of PBL

The majority of lymphocytes in the blood are in resting state (G_0 -phase). Activation of lymphocytes induces their transformation to lymphoblasts and the commitment to the cell cycle [29]. The lymphoblast population can be discriminated from resting lymphocytes by a cytofluorimetric analysis using side-scatter (granularity) and forward-scatter (size) parameters. We have previously demonstrated that mildly oxLDLs (over 10 μ g/ml) inhibited the [3H]thymidine uptake by activated T-lymphocytes without cell-death increase [9]. As shown in Figure 1(A), the inhibition is correlated with a decrease in the blast population. This effect was time- and concentration-dependent (Figures 1A and 1B). In addition, the mitogenic response to PHA of PBL preincubated for 24 h with oxLDLs (10, 50 and 100 μ g/ml) and then washed was not inhibited and was similar to that of cells preincubated with native LDLs, suggesting that oxLDLs are not toxic on resting lymphocytes after 24 h incubation. The preincubation with oxLDLs did not decrease the sensitivity of lymphocytes to PHA stimulation (Figure 1C). Finally, experiments indicated that a co-incubation for 24 h of PBL with PHA + oxLDLs is sufficient to induce an inhibitory effect on cell proliferation (Figure 1D), suggesting that oxLDLs can alter the events triggering the DNA synthesis in activated T-lymphocytes during the first 24 h. Among the first steps of activation is the binding of PHA to the cell surface of T-lymphocytes. We have previously demonstrated that the inhibition is not due to interference of oxLDLs with the binding of PHA [9].

Effect of oxLDLs on IL-2 secretion of T-lymphocytes

As shown in Figure 2A, when lymphocytes were activated by PHA the IL-2 concentration rose sharply during the first 24 h and decreased afterwards in supernatants. The presence of native LDLs (100 and 200 μ g/ml) maintained (or increased) the IL-2 level. In contrast, incubation with oxLDLs (100 or 200 μ g/ml) induced a significant decrease of IL-2 concentration as soon as 24 h after activation. Moreover, this inhibition was directly correlated with the TBARS and hydroperoxide levels of oxLDLs (results not shown). The comparison of UV-oxLDLs with 2 h-Cu-oxLDLs, characterized by a similar mild oxidation level, indicated that the inhibition of IL-2 secretion (Figure 2B) or of PHA-induced proliferation (results not shown) was independent of the oxidation methods.

For examining the earlier effect of oxLDLs, the intracytoplasmic IL-2 level was analysed by cytofluorimetry. In PHA-activated lymphocytes, IL-2 $^+$ CD3 $^+$ cells were not detectable

Figure 1 Effect of oxLDLs on the blast transformation and the proliferation of activated lymphocytes

(A) PHA-activated PBL were incubated in triplicate cultures with 50 and 100 μ g/ml of native LDLs (open symbols) or oxLDLs (closed symbols) for 72 h in RPMI/2% LPDS. The percentage of the blast population (squares) was estimated by cytofluorimetry (% of blast $< 2\%$ in unstimulated PBL). Cell proliferation (circles) was determined by [3H]thymidine uptake (c.p.m. < 500 in unstimulated PBL). The results are the mean $\pm$ S.D. for three independent experiments performed with PBL from different donors. (B) PBL were activated by PHA in the absence (*) or presence of 50 μ g/ml ($\square$) and 100 μ g/ml ($\circ$) native LDLs or 50 μ g/ml ($\blacksquare$) and 100 μ g/ml ($\bullet$) oxLDLs for 24, 48 and 72 h in RPMI/2% LPDS. Results are expressed as means of triplicate determinations of blast-cell percentage. A second experiment performed with a different donor gave identical results. (C) PBL in triplicate cultures were (i) co-incubated for 96 h with PHA + 10, 50 and 100 μ g/ml of native LDLs ($\circ$) or oxLDLs ($\bullet$), (ii) pre-incubated for 24 h with 10, 50 and 100 μ g/ml of native LDLs ($\square$) or oxLDLs ($\blacksquare$), washed three times, activated by PHA and cultured for 72 h. Proliferation was estimated by determination of [3H]thymidine uptake. Results are expressed as percentage of the control and are means for two independent experiments performed with PBL from different donors. (D) PBL were co-incubated with PHA and 10, 50 and 100 μ g/ml oxLDLs for 24 h, washed three times and incubated in the absence (squares) or in the presence (circles) of oxLDLs for 48 h. Results are expressed as c.p.m.. A second experiment performed with PBL from a different donor gave similar results.

before 8 h by flow cytometry (results not shown). So we have used a stronger stimulator of IL-2 secretion, i.e. PMA + ionomycin. When PBL were activated by PMA + ionomycin, the concentration of IL-2 in the supernatant was much higher than that observed after PHA activation (Figure 2C). However, the inhibitory effect of oxLDLs was very similar to that observed in the case of activation by PHA and was already appreciable by 24 h. Cytofluorimetric analysis detected a significant signal as soon as 4 h after stimulation: $17 \pm 4\%$ of IL-2 $^+$ CD3 $^+$ positive cells (versus $0.6 \pm 0.3\%$ in unactivated-CD3 $^+$ lymphocytes). As shown in Figure 2(D), the IL-2 $^+$ T-cell percentage significantly decreased in the presence of oxLDLs (50 and 100 μ g/ml) as compared with cells incubated with LDLs.

To determine whether the decrease of IL-2 secretion induced by oxLDLs can directly be involved in the inhibition of proliferation, we added recombinant IL-2 to the cultures. As shown in Figure 3, the addition of IL-2 stimulated DNA synthesis when PHA-activated lymphocytes were cultured without or with oxLDLs for 72 h. The proliferation increased as a function of the IL-2 concentration in control cultures and in cultures with 50 μ g/ml oxLDLs, but seemed to reach a limit when the activated lymphocytes were cultured with 100 μ g/ml oxLDLs. The same results were obtained when IL-2 was added 24 h

Figure 2 Effect of oxLDLs on the early IL-2 production in activated T-lymphocytes

(A) PBL were activated by PHA in the absence (✱) or presence of 100 µg/ml (□) and 200 µg/ml (○) native LDLs or 100 µg/ml (■) and 200 µg/ml (●) oxLDLs in RPMI/2% LPDS. IL-2 was detected by an ELISA method in culture supernatants after 24, 48 and 72 h of culture (IL-2 < 0.8 pg/ml in unstimulated PBL at 24, 48 or 72 h). Results are the means ± S.D. from four independent experiments performed with lymphocytes from different donors. (B) PHA-activated PBL were incubated for 24 h in RPMI/2% LPDS with 100 µg/ml or 200 µg/ml native LDLs (nat, □), UV-oxidized (UV, ■) or 2 h-Cu-oxidized (Cu, ▨) LDLs and IL-2 was measured in the culture supernatants. Results are shown as percentage of the PHA-activated control without LDLs and are means for three independent experiments, each performed with a different donor (**P* < 0.05 versus control cells, Student's *t* test). (C) IL-2 was detected in the culture supernatants of PBL activated by PMA + ionomycin in the absence (✱) or presence of 100 µg/ml (□) and 200 µg/ml (○) native LDLs or 100 µg/ml (■) and 200 µg/ml (●) oxLDLs for 48 h. Results are means for two independent experiments performed with lymphocytes from different donors. (D) PBL were activated by PMA + ionomycin in the presence of 50 µg/ml and 100 µg/ml native LDLs (□) or oxLDLs (■) for 4 h in RPMI/2% LPDS. Intracellular IL-2 was detected in CD3⁺ lymphocyte population by cytofluorimetry. The percentage of IL-2⁺CD3⁺ cells was determined. Results are expressed as the percentage of control without LDLs and are the mean ± S.D. for five independent experiments performed with lymphocytes from different donors (**P* < 0.05 versus control cells or LDL-treated cells, Student's *t* test).

after the beginning of activation (results not shown). In agreement with our previous data, a clear inhibition of CD25 expression was observed at the surface of CD3⁺ lymphocytes incubated with 50 and 100 µg/ml oxLDLs (Figure 3, inset). The decreased T-cell proliferation is thus partly explained by a relative lack of IL-2 and a decreased IL-2 receptor expression at the surface of the lymphocytes.

Effect of oxLDLs on the binding of NF-κB to DNA in activated PBL

The results mentioned above suggest that oxLDLs may act before 4 h on events induced by T-lymphocyte stimulation. The activation of T-lymphocytes by antigens and mitogens triggers the early *trans*-activation of the IL-2 gene, which depends on the binding of NF-κB to specific DNA sites. We have examined the translocation of NF-κB to the nucleus by EMSA after activation of PBL by PHA or PMA + ionomycin. In agreement with previous studies [28,30,31], nuclear extracts from resting lymphocytes contained a constitutive nuclear band corresponding to p50/p50 homodimers (Figure 4A). PHA or PMA + ionomycin

Figure 3 Addition of IL-2 in cultures of PHA-activated lymphocytes co-incubated with oxLDLs

PHA-activated PBL were cultured in triplicate with 50 µg/ml and 100 µg/ml of oxLDLs in the absence (■) or in the presence of 50 units/ml (4000 pg/ml) (▨) and 200 units/ml (16 000 pg/ml) (□) of recombinant IL-2 for 72 h in RPMI/2% LPDS. Cell proliferation was estimated by determination of [³H]thymidine uptake. Results are means of triplicate determinations. One other separate experiment, performed with a different donor, gave identical results. In the inset is shown the expression of CD25 in PHA-activated CD3⁺ T-lymphocytes cultured for 72 h with oxLDLs. The results are expressed as CD25 mean fluorescence intensity (MF) in double-positive populations (CD3⁺25⁺).

activation induced the decrease of the p50/p50 homodimer band and the emergence of a new retarded band corresponding to the binding of the oligonucleotide with the p65/p50 heterodimer. This later dimer is involved in the activation of IL-2 and CD25 genes after stimulation of T-cells with antigens, anti-CD3 antibodies, PHA or PMA + ionomycin [28,30,31]. When lymphocytes were exposed simultaneously to oxLDLs and activator, the intensity of this last band decreased whereas the p50/p50 homodimer band was unchanged. Figure 4(B) shows the specificity of the p65/p50 heterodimer as supershifts revealed by a preincubation with specific anti-p65 and anti-p50 antibodies and competition with an irrelevant unlabelled oligonucleotide (AP1) and the specific unlabelled NF-κB oligonucleotide. Since exposure to oxLDLs seemed to inhibit the NF-κB activation induced by PHA or PMA + ionomycin in lymphocytes, the role of the inhibitor IκB-α was examined in the same experimental conditions. As expected, lymphocyte activation led to a rapid proteolytic removal of IκB-α in the absence of lipoproteins. This proteolysis was not modified by LDLs, whereas oxLDLs clearly blocked the cytoplasmic depletion of IκB-α (Figure 4C).

DISCUSSION

The present data demonstrate that mildly oxLDLs inhibit the blastic transformation of PHA-activated lymphocytes. This is in agreement with our previous work showing an inhibition of DNA synthesis and an accumulation of cells at the G₁-phase/S-phase interface in activated T-lymphocytes [9]. While the 24 h preincubation of naïve lymphocytes with oxLDLs did not inhibit

Figure 4 Effect of oxLDLs on activation of NF-κB in stimulated lymphocytes

(A) At the left, PBL were activated by PHA for 60 min in the absence (lane 2) or the presence of 100 $\mu\text{g}/\text{ml}$ native LDLs (lane 3) or oxLDLs (lane 4). At the right, PBL were activated by PMA + ionomycin for 15 min without (lane 6) or with 100 $\mu\text{g}/\text{ml}$ native LDLs (lane 7) or oxLDLs (lane 8). In lanes 1 and 5 are unstimulated PBL. Nuclear p65p50 and p50p50 dimers were detected by EMSA with the consensus-specific NF-κB oligonucleotide labelled with [γ - ^{32}P]ATP. The data are representative of three independent experiments performed with lymphocytes from different donors. (B) PBL were activated by PMA + ionomycin for 15 min. Nuclear NF-κB proteins were detected directly with the consensus-labelled NF-κB oligonucleotide (lane 1) or after a 30 min preincubation of nuclear proteins with 1 μg of specific anti-p65 (lane 2) or anti-p50 (lane 3) antibodies, 100-fold molar excess of unlabelled irrelevant AP1 oligonucleotide (lane 4) or 100-fold molar excess of unlabelled NF-κB oligonucleotide (lane 5). The data are representative of two independent experiments performed with lymphocytes from different donors. Similar results (not shown) were observed after PHA activation. (C) The PBL were activated in the same way as described for (A). The cytoplasmic IκB-α protein (IκB) and the control β-actin were detected by Western blot using a specific antibody. The data are representative of three independent experiments performed with lymphocytes from different donors.

the subsequent proliferative response to PHA, a similar incubation of activated lymphocytes induced a maximal inhibition, suggesting that the sensitivity to oxLDLs depends on the activation state of lymphocytes (Figure 1D). We have previously shown that oxLDLs inhibit the expression of CD25 24 h after lymphocyte activation by PHA, whereas the expression of CD69 and HLA-DR is unchanged. The present work demonstrates that IL-2 synthesis was also inhibited by oxLDLs. This effect is time- and dose-dependent and correlates with the level of LDL oxidation at the time of activation. The decrease in intracytoplasmic IL-2 level in CD3⁺ T-lymphocytes can be demonstrated as soon as 4 h after stimulation. Taken together, these data suggest that the suppressive effect of oxLDLs on T-lymphocyte proliferation is a consequence of the inhibition of IL-2 secretion and of the impairment of CD25 expression, since the interaction of IL-2 with its receptor is usually required for activated T-lymphocytes to progress from the G₁- to the S-phase of the cell cycle [29].

During T-cell activation, the expression of IL-2 and CD25 genes is one of the earliest events. This gene expression involves several identified nuclear complexes such as NF-κB, AP-1, NF-AT [25,29–31]. The present data strongly suggest that oxLDLs might inhibit the expression of the two proteins (IL-2 and CD25), because they alter, at least partly, the transcription mechanisms involving the NF-κB family. Indeed, oxLDLs (100 $\mu\text{g}/\text{ml}$) down-regulated the activation-induced binding of p65/p50 heterodimers to a consensus sequence of DNA and maintained the nuclear presence of p50 dimers probably involved in the repression of IL-2-gene transcription [25]. Since κB elements have been found on the promoters of several other cytokine genes in lymphocytes and monocytes, the inhibition of the NF-κB system might also be involved in the oxLDL-induced impairment of production of interferon-γ (known to be synthesized by activated T-lymphocytes) [8] and of IL-1β and tumour necrosis factor-α (produced by activated monocytes) previously reported [6]. The effect on NF-κB activation was

observed immediately after lymphocyte stimulation, but we cannot exclude the possibility that oxLDLs alter NF-κB activation at later time points and thus dysregulate the control of several genes encoding other growth-regulating proteins [30,31]. Indeed, T-cell activation induces an early decrease in the level of p50 dimers in the nucleus and a concomitant increase in the level of active p50/p65 heterodimers [24,25,28,30,31], whereas in the late phase (from 24 to 72 h after activation) the expression of the p50 NF-κB protein is increased progressively [32].

Our data demonstrate that oxLDLs inhibit the activation-induced depletion of the IκB-α protein immediately after stimulation. In resting lymphocytes, IκB-α is bound to the p50/p65 heterodimer in the cytoplasm. When lymphocytes are exposed to inducers of NF-κB, IκB-α is rapidly phosphorylated and degraded by the proteasome, whereas the heterodimer is translocated [26,30]. Our results suggest that the NF-κB inhibition could be explained either by an absence of IκB degradation (e.g., decrease of its proteolysis by the proteasome) or by a dysregulation of its phosphorylation.

Using immunofluorescence and immunohistochemical techniques, activated NF-κB has been detected in atherosclerotic lesions in smooth-muscle cells, macrophages and endothelial cells [33]. Several recent studies have shown that, in these various cell types, the NF-κB system can react differently to oxLDLs. According to the level of oxidation of LDLs, the cell type and/or the activation state of the cells, oxLDLs may activate or inhibit NF-κB [3,6,34,35]. For instance, a short exposure to oxLDLs can activate NF-κB in resting monocytes and be accompanied by an increase of IL-8-promoter-dependent transcription [3]. In contrast, preincubation or co-incubation of lipopolysaccharide (LPS)-activated monocytes with oxLDLs inhibit NF-κB activation [3,6,35]. Similarly, in vascular smooth-muscle cells, oxLDLs inhibit LPS-induced activation of NF-κB [36]. In agreement with these data, the results of the present study suggest that oxLDLs may suppress the activation of NF-κB factors in lymphocytes, leading to the subsequent inhibition of

IL-2 synthesis and IL-2 receptor expression. However, several other transcription factors control the IL-2 and CD25 gene expression in activated lymphocytes and could be differently altered by oxLDLs. A recent study using human monocytes-macrophages showed that oxLDLs activate transcription factor AP1 [37]. Another study demonstrated that oxLDLs have similar inhibitory effects on LPS-induced activation of NF- κ B and AP1 in macrophages [6], whereas others suggest that they have opposing effects on the two transcription factors [36]. Preliminary results in our laboratory indicate that oxLDLs might induce a moderate down-modulation of AP1 binding to DNA after PHA or PMA + ionomycin-mediated activation.

Several signal-transduction pathways may lead to the activation of NF- κ B proteins in T-lymphocytes. For example, PKC- and Ca²⁺-dependent pathways participate in the regulation of NF- κ B activation [38]. oxLDLs are known to be able to induce various intracellular transduction signals in endothelial cells, smooth-muscle cells and macrophages [35,39–42]. Experiments are in progress with inhibitors to assess the putative modulating effect of oxLDLs on various signalling processes.

It is not known which factor in oxLDLs is responsible for the observed effect on lymphocytes. Under our experimental conditions, oxLDLs are mainly oxidized at the lipid level and are taken up by lymphocytes via LDL receptors [27], and lipid oxidation is characterized by no significant modification of lysophosphatidylcholine content, and a moderate increase of oxysterols and hydroperoxides [9,43]. It has been suggested that lipid oxidation products have an important biological effect on transcriptional regulation. For instance, oxysterols inhibit LPS-induced activation of NF- κ B in macrophages and smooth-muscle cells [6,36], and it can be proposed that lipid oxidation products such as oxysterols and hydroperoxides may be involved in the inhibition of the T-cell activation.

The present results indicate that mildly oxLDLs may dysregulate the NF- κ B system and inhibit IL-2 production in lymphocytes. Taken together with our previous observations [9], these experiments suggest that oxLDLs may inhibit early steps of the T-lymphocyte activation. In atherosclerosis, the oxLDLs might alter the lymphocyte activation *in situ* or minimize the response of activated T-lymphocytes, which migrate toward the lesions. The role of this potential immunosuppressive effect on the progression of the atherosclerotic lesions remains to be demonstrated *in vivo*. Since recent reports suggest that common chronic infections may contribute to the pathogenesis of coronary atherosclerosis [44], oxLDLs might impair the immune response against the infections of the vascular wall and thus be involved in the progression of the atherosclerotic lesions.

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REFERENCES

- Goldstein, J. L. and Brown, M. S. (1977) *Annu. Rev. Biochem.* **46**, 897–930
- Witztum, J. L. and Steinberg, D. (1991) *J. Clin. Invest.* **88**, 1785–1792
- Brand, K., Eisele, T., Kreuzel, U., Page, M., Page, S., Haas, M., Gerling, A., Kaltschmidt, C., Neumann, F. J., Mackman, N. et al. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17**, 1901–1909
- Frostegard, J., Wu, R., Giscombe, R., Holm, G., Lefvert, A. K. and Nilsson, J. (1992) *Arterioscler. Thromb.* **12**, 461–467
- Uyemura, K., Demer, L. L., Castle, S. C., Jullien, D., Berliner, J. A., Gately, M. K., Warrier, R. R., Pham, N., Fogelman, A. M. and Modlin, R. L. (1996) *J. Clin. Invest.* **97**, 2130–2138
- Ohlsson, B. G., Englund, M. C., Karlsson, A. L., Knutsen, E., Erixon, C., Skribeck, H., Liu, Y., Bondjers, G. and Wiklund, O. (1996) *J. Clin. Invest.* **98**, 78–89
- Stemme, S., Faber, B., Holm, J., Wiklund, O., Witztum, J. L. and Hansson, G. K. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3893–3897
- Viora, M., Straface, E., Di Genova, G., Fattorossi, A., Rivabene, R., Camponeschi, B., Masella, R. and Malorni, W. (1997) *Biochem. Biophys. Res. Commun.* **232**, 359–363
- Caspar-Bauguil, S., Saadawi, M., Nègre-Salvayre, A., Thomsen, M., Salvayre, R. and Benoist, H. (1998) *Biochem. J.* **330**, 659–666
- Dansky, H. M., Charlton, S. A., Harper, M. M. and Smith, J. D. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4642–4646
- Daugherty, A., Pure, E., Delfel-Butteiger, D., Chen, S., Leferovich, J., Roselaar, S. E. and Rader, D. J. (1997) *J. Clin. Invest.* **100**, 1575–1580
- Emeson, E. E., Shen, M. L., Bell, C. G. and Qureshi, A. (1996) *Am. J. Pathol.* **149**, 675–685
- Gupta, S., Pablo, A. M., Jiang, X., Wang, N., Tall, A. R. and Schindler, C. (1997) *J. Clin. Invest.* **99**, 2752–2761
- Drew, A. F. and Tipping, P. G. (1995) *Atherosclerosis* **116**, 181–189
- Zhou, X., Stemme, S. and Hansson, G. K. (1996) *Am. J. Pathol.* **149**, 359–366
- Emeson, E. E. and Shen, M. L. (1993) *Am. J. Pathol.* **142**, 1906–1915
- Fyfe, A. I., Qiao, J. H. and Lusis, A. J. (1994) *J. Clin. Invest.* **94**, 2516–2520
- Hansson, G. K., Holm, J., Holm, S., Fotev, Z., Hedrich, H. J. and Fingerle, J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10530–10534
- Roselaar, S. E., Schonfeld, G. and Daugherty, A. (1995) *J. Clin. Invest.* **96**, 1389–1394
- Nilsson, J., Calara, F., Regnstrom, J., Hultgardh-Nilsson, A., Ameli, S., Cercek, B. and Shah, P. K. (1997) *J. Am. Coll. Cardiol.* **30**, 1886–1891
- Ross, R. (1993) *Nature (London)* **362**, 801–809
- Wick, G., Schett, G., Amberger, A., Kleindienst, R. and Xu, Q. (1995) *Immunol. Today* **16**, 27–33
- Stemme, S. and Hansson, G. K. (1994) *Coron. Artery Dis.* **5**, 216–222
- Baeuerle, P. A. and Henkel, T. (1994) *Annu. Rev. Immunol.* **12**, 141–179
- Jain, J., Loh, C. and Rao, A. (1995) *Curr. Opin. Immunol.* **7**, 333–342
- Maniatis, T. (1997) *Science* **278**, 818–819
- Nègre-Salvayre, A., Paillous, N., Dousset, N., Bascoul, J. and Salvayre, R. (1992) *Photochem. Photobiol.* **55**, 197–204
- Kanno, T. and Siebenlist, U. (1996) *J. Immunol.* **157**, 5277–5283
- Kelly, K. and Siebenlist, U. (1995) *Curr. Opin. Immunol.* **7**, 327–332
- Kahn-Perles, B., Lipsey, C., Lecine, P., Olive, D. and Imbert, J. (1997) *J. Biol. Chem.* **272**, 21774–21783
- Pimentel-Muinos, F. X., Mazana, J. and Fresno, M. (1995) *Eur. J. Immunol.* **25**, 179–186
- Yu, X. P., Bellido, T. and Manolagas, S. C. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10990–10994
- Brand, K., Page, S., Rogler, G., Bartsch, A., Brandl, R., Knuechel, R., Page, M., Kaltschmidt, C., Baeuerle, P. A. and Neumeier, D. (1996) *J. Clin. Invest.* **97**, 1715–1722
- Maziere, C., Auclair, M., Djavaheri-Mergny, M., Packer, L. and Maziere, J. C. (1996) *Biochem. Mol. Biol. Int.* **39**, 1201–1207
- Schackelford, R. E., Misra, U. K., Florin-Casteel, K., Thai, S. F., Pizzo, S. V. and Adams, D. O. (1995) *J. Biol. Chem.* **270**, 3475–3478
- Ares, M. P., Kallin, B., Eriksson, P. and Nilsson, J. (1995) *Arterioscler. Thromb. Vasc. Biol.* **15**, 1584–1590
- Jovinge, S., Ares, M. P., Kallin, B. and Nilsson, J. (1996) *Arterioscler. Thromb. Vasc. Biol.* **16**, 1573–1579
- May, M. J. and Ghosh, S. (1998) *Immunol. Today* **19**, 80–88
- Matsumura, T., Sakai, M., Kobori, S., Biwa, T., Takemura, T., Matsuda, H., Hakamata, H., Horiuchi, S. and Shichiri, M. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17**, 3013–3020
- Kusuhara, M., Chait, A., Cader, A. and Berk, B. C. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17**, 141–148
- Escargueil-Blanc, I., Salvayre, R. and Nègre-Salvayre, A. (1994) *FASEB J.* **8**, 1075–1080
- Auge, N., Andrieu, N., Nègre-Salvayre, A., Thiers, J. C., Levade, T. and Salvayre, R. (1996) *J. Biol. Chem.* **271**, 19251–19255
- Schmitt, A., Nègre-Salvayre, A., Trolly, M., Valdiguié, P. and Salvayre, R. (1995) *Biochim. Biophys. Acta* **1256**, 284–292
- Chiu, B., Viira, E., Tucker, W. and Fong, I. W. (1997) *Circulation* **96**, 2144–2148