Oxidized low density lipoproteins induce apoptosis in PHA-activated peripheral blood mononuclear cells and in the Jurkat T-cell line

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Abstract **Oxidized low density lipoproteins (oxLDLs) and activated T lymphocytes are present in early atherosclerotic plaques. It has been shown that oxLDLs are cytotoxic to cultured vascular cells but their possible toxic action on T lymphocytes has not been described. Peripheral blood lymphocytes from healthy individuals were stimulated in vitro with the polyclonal activator phytohemagglutinin and treated with various doses of native and mildly oxidized LDLs. Low doses of oxLDLs inhibited cell growth and DNA synthesis after 48 h culture and at 200 μg apoB/ml we observed a loss of cell viability. Dead cells did not exhibit significant increase of alteration of membrane integrity (i.e., necrosis) but showed chromatin fragmentation evaluated by DNA staining with 4′,6-diamidino-2-phenylindole and propidium iodide. This fragmentation increased with TBARS and hydroperoxide levels. The expression of early apoptosis marker Apo2.7 rose among the $CD3^+$ T-cell population. In addition, morphological analysis showed apoptotic features (cell shrinking, nucleus condensation, and fragmentation). Study of phosphatidylserine expression using Annexin V confirmed that oxLDLs induced apoptosis in activated lymphocytes. In the Jurkat T-cell line cultured with oxLDLs, apoptotic morphological changes (condensation and nucleus fragmentation) were observed and they were accompanied by DNA fragmentation visualized by propidium iodide staining and electrophoresis showing apoptotic ladder. These results demonstrate that mildly oxidized LDLs induce apoptosis in a part of activated and proliferating T cells. T-lymphocyte apoptosis induction in atherosclerotic lesions might contribute to the development of an unappropriate local T cell response.**—Alcouffe, J., S. Caspar-Bauguil, V. Garcia, R. Salvayre, M. Thomsen, and H. Benoist. **Oxidized low density lipoproteins induce apoptosis in PHA-activated peripheral blood mononuclear cells and in the Jurkat T-cell line.** ***J. Lipid Res.*** **1999.** 40: **1200–1210.**

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Oxidized low density lipoproteins (oxLDLs) detected in atherosclerotic plaques are thought to play an important role in the development of atherosclerosis (1, 2). Their role in the progress of lesions is suggested by their various biological effects, both on vascular wall cells and on cells infiltrating the lesions (3–7). For instance, oxLDLs modulate monocyte recruitment and activation and promote the formation of foam cells and fatty streaks, considered to be the first step in plaque initiation. In addition, oxLDLs are cytotoxic to many cell types and are probably implicated in the death of vascular wall cells, leading to plaque instability (8, 9). Several studies in vitro indicate that oxLDLs trigger apoptosis in cultured cell types such as smooth muscle cells (SMCs), endothelial cells, fibroblasts, and macrophages (10–12). The early lesions are hypercellular but become cytopenic when plaque cells undergo necrosis or apoptosis, suggesting that cell death occurs during the disease pathogenesis and could contribute to lesion development (9).

Activated T-cells are present in early atherosclerotic areas and may constitute up to 30% of cells in the lesion (13–15). This indicates that atherosclerotic lesions may be sites of specific T-cell-mediated immune response and not only a "nonspecific" inflammatory reaction (13, 16, 17). Some data obtained in animals suggest that immune mechanisms and T-cell presence may be protective factors, at least during the early stages of atherogenesis (18–20), whereas other works mention disease aggravation as a consequence of an increased activity of inflammatory cells inside the vessel wall (21, 22). Whether the immune response represents a primary or a secondary event in atherosclerosis remains to be clarified. Lymphocytes constitute a highly regulated cell population, producing various cytokines able to act on vascular wall cells. It is thus quite

Abbreviations: PBMC, peripheral blood mononuclear cells; LDLs, low density lipoproteins; oxLDLs, oxidized LDLs; PI, propidium iodide; MGG, May Grünwald Giemsa; PHA-P, phytohemagglutinin P; ROI, reactive oxygen intermediates; apoB, apolipoprotein B; TBARS; thiobarbituric acid-reactive substances; DAPI, 4′,6-diamidino-2-phenylindole.

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logical to suggest that lymphocyte death within the plaques may have consequences on the evolution of the atheroma.

LDL oxidation is a progressive process leading at first to mildly oxidized LDLs, characterized by a relatively low content of lipid peroxidation derivatives and only slight apolipoprotein B (apoB) modifications (23, 24). Exposure to ultraviolet (UV) radiation is an easy way to obtain mildly oxidized LDLs (25). Many anti-oxidative mechanisms prevent or delay LDL oxidation in vivo and mildly oxidized LDLs can thus be representative of the initial step of the LDL oxidative attack. Mildly oxidized LDLs exhibit various dose-dependent biological effects (4, 6, 26) and as they are taken up by the apoB/E receptor, they can act directly on lymphocytes.

Recent observations have demonstrated that apoptosis was common in inflammatory cells, i.e. macrophages and T lymphocytes, in atherosclerotic lesions (27). The mechanisms of T-lymphocyte apoptosis are poorly understood in the plaques. To our knowledge, oxLDL apoptotic effects have never been investigated on T lymphocytes, which are the main lymphocyte population present in atherosclerotic lesions. The present study was designed to investigate oxLDL effects on T cell survival under activation and proliferation conditions.

MATERIALS AND METHODS

Cell culture and T-cell activation

All cells were cultured in RPMI 1640 medium containing Glutamax I, supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mm), and 10% heat-inactivated fetal calf serum (FCS) (Gibco, Cergy-Pontoise, France). During assays, FCS was replaced by 2% lipoprotein-depleted serum (LPDS).

Experiments were carried out on three different lymphocyte populations: the human T lymphoma cell line Jurkat (ATCC, Manassas, VA), an Epstein Barr virus transformed B-cell line (B.B.), and peripheral blood mononuclear cells (PBMC). PBMC were separated from heparinized venous blood from healthy donors by centrifugation (20 min, 600 *g*) over Ficoll (Gibco) and activated by phytohemagglutinin P (PHA-P, 1 μg/ml, Sigma-Merck, Darmstadt, Germany) that stimulates T lymphocytes.

Cell viability was determined by trypan blue exclusion test and exceeded 90% before all experiments.

LDL isolation and oxidation

LDLs (d 1.019–1.063) and LPDS were isolated from pooled fresh human sera by sequential ultracentrifugation as previously described (28). LDLs were dialyzed against 150 mm NaCl containing 0.3 mm EDTA, sterilized by filtration (0.2 μm Millipore membrane) and stored at 4°C under nitrogen until use (up to 2 weeks). Under the standard conditions, LDLs were oxidized as follows: apolipoprotein B (apoB) solution (2 mg/ml) was exposed to UV-C radiation (254 nm; 0.5 mW/cm^2) during 2–2.5 h (28). In some experiments LDLs were oxidized during various time periods (from 1 h to 4 h) as indicated. LDL oxidation state was estimated by determination of: *1*) thiobarbituric acid-reactive substances (TBARS) by the fluorometric method of Yagi (29); *2*) hydroperoxide concentrations by use of a colorimetric commercial kit (PeroXOquant; Pierce, Rockford, IL) based on the oxidation of ferrous to ferric ion in the presence of xylenol orange (30). Under the standard conditions, oxLDLs (2–2.5 h UV radiation) contained 4.5 ± 0.9 nmol of TBARS/mg of apoB (versus 0.4 ± 0.1 for native LDLs) and 78.7 ± 7.4 nmol of hydroperoxides/mg of apoB (versus 13.2 ± 2.1 for native LDLs). When native LDLs were incubated with activated PBMC for 72 h, the oxidation level increased from 0.5 ± 0.2 nmol of TBARS/mg of apoB at t = 0 to 1.1 ± 0.2 at t = 72 h. The changes observed with oxLDLs were about the same.

Proliferative assays

Cell proliferation was evaluated by MTT assay (Boehringer Mannheim, Germany) according to the manufacturer's instructions. After cultures at various times and conditions, cells were seeded in 96-well flat-bottom plates (2×10^5 cells/ml, 100 μl/well) containing 0.5 μg/μl MTT labeling reagent/well for 4 h. Solubilization buffer was added and plates were incubated overnight at 37°C so that the formazan crystals could be solubilized. The obtained products were spectrophotometrically quantified using an ELISA reader (λ = 540 nm). Alternatively, the cell number was evaluated by light microscopy in the presence of trypan blue.

DNA synthesis was evaluated by [^{3}H]thymidine uptake. Cells (10^5/ml) in RPMI 1640 medium supplemented with 2% LPDS were seeded in 96-well round-bottom plates (100 μl/well) and incubated with LDLs at 37°C. Each well was labeled with 1 μCi [^{3}H]thymidine (1 Ci = 37 Gbq, ICN, Orsay, France) during the last 16 h of culture. Cells were harvested on glass fiber filters by means of an automatic cell harvester (Harvester 96 Tomtec, Wallac-EG&G instruments, Evry, France) and the amount of incorporated [^{3}H]thymidine (CPM) was determined on Microbeta trilux (Wallac-EG&G instruments). Triplicate culture mean radioactivity was used for calculation.

Evaluation of necrosis and apoptosis

Determination of chromatin fragments by fluorometric method. After incubation periods, cells were lysed 15 min in 500 μl lysis buffer (5 g/l Triton X-100, 20 mmol/l EDTA, 5 mmol/l Tris, pH = 8) and then ultracentrifuged (23,000 rpm, 20 min) to separate the chromatin pellet from cleavage products (31). The pellet (resuspended in 500 μl 10 mmol/l Tris buffer, containing 1 mmol/l EDTA, pH = 8) and supernatant DNA content were determined by fluorometric procedure according to the method of Kapuscinski and Skoczylas (32) and using 4′,6-diamidino-2-phenylindole (DAPI).

Flow cytofluorometric analysis. DNA fragmentation level in LDL-treated cells was investigated using propidium iodide (PI) added to permeabilized cells, as previously described (33). The fluorescence intensity from stained cell nuclei is proportional to the cellular DNA content. The nuclei were analyzed by the multicycle A. V. Phoenix flow system (P. F. Rabinovitch, University of Washington, Seattle, WA).

The percentage of apoptotic cells was evaluated by the detection of the Apo2.7 antigen and phosphatidylserine (PS) whose expression appears to be restricted to cells undergoing apoptosis. To determine apoptosis in T lymphocytes, PHA-activated PBMC were surface labeled with phycoerythrin-conjugated anti-Apo2.7 and fluorescein isothiocyanate (FITC)-conjugated anti-CD3 monoclonal antibodies (Immunotech, Marseille, France). Alternatively, activated PBMC were stained with Annexin V-FITC which binds preferentially to PS (Annexin V-FITC kit, Immunotech, Marseille, France) and with PI which detects loss of plasma membrane integrity (i.e., necrosis). Four distinct phenotypes become distinguishable: *i*) the viable population (annexin V and PI negative cells), *ii*) the apoptotic population (annexin V positive/PI negative cells), *iii*) the necrotic population (annexin V negative/PI positive cells), and *iv*) the secondary necrotic population (annexin V positive/PI positive cells). In some experiments, the percentage of necrotic cells was evaluated after cell incubation in phosphate buffer solu-

tion (PBS) with PI 5 μg/ml alone. Plasma membrane integrity was controlled by the absence of PI fluorescence in lymphocyte populations.

Whatever the staining technique used, ten thousand events were collected and analyzed on a Coulter Elite cytofluorimeter.

Microscopical analysis. Necrosis was evaluated by the trypan blue exclusion test and by determining lactate dehydrogenase released in the culture medium (Roche assay kit. MA kit 10). Alternatively, necrosis and apoptosis were evaluated by using a fluorescent microscope, after concomitant fluorescent staining by two vital fluorescent dyes, 0.6 mm SYTO-13 (a permeant DNA intercaling yellow-colored probe) and 15 mm propidium iodide (a non-permeant intercaling red probe). Viable lymphoblasts exhibited nuclei with loose chromatin colored yellow by SYTO. Necrotic cells displayed red nuclei. Apoptotic nuclei had condensed and/or fragmented chromatin colored yellow whereas post-apoptotic nuclei were red colored with apoptotic features.

The cell morphology was also examined with a Leica Diaplan light microscope after cytocentrifugation (5 min, 124 *g*) and staining with May Grünwald-Giemsa (MGG) dye.

Electrophoretic analysis of DNA fragmentation

Genomic DNA was extracted by using the G-nome kit (Bio 101, La Jolla, CA). DNA samples were loaded onto 1.8% agarose ethidium bromide-stained gels and run for 1 h at 50 V. The 100 bp DNA ladder from Promega (Madison, WI) was used as molecular weight marker.

RESULTS

Oxidized LDLs inhibit PHA-activated PBMC proliferation and induce no necrosis

In a previous work, we have shown that mildly oxidized LDLs inhibited activated T-lymphocyte (oligoclonal and monoclonal populations) proliferation with no significant induction of necrosis at doses between 10 to 100 μg apoB/ml (34). The present study confirms the significant inhibitory effect on the heterogeneous population of PHA-activated PBMC. Compared to control, incubation with 50–200 μg apoB/ml native LDLs induced a moderate decrease of number of cells able to reduce MTT after 72 h culture (**Fig. 1A**), but allowed cell growth over this period (Fig. 1B). In contrast, 200 μg apoB/ml oxLDLs induced a clear growth arrest after 48 h culture (Fig. 1B) which was confirmed by the significant decrease of number of trypan blue-negative cells (Fig. 1C). The cell number reduction could be due to oxLDL inhibitory effect on cell proliferation and/or to a cell death increase. In the presence of 200 μg apoB/ml oxLDLs, DNA synthesis (evaluated by [^{3}H]thymidine incorporation) was almost stopped (Fig. 1E). No significant loss of plasma membrane integrity was observed, as assessed by the trypan blue exclusion test (Figs. 1D and 1E, inset), thus suggesting that the O.D. decrease in the MTT test, the cell number reduction, and the inhibition of [^{3}H]thymidine incorporation are not due to necrosis. In addition, the determination of LDH released into culture medium after 72 h incubation with 200 μg/ml oxLDLs (3 h UV-radiation) did not show any significant increase as compared to cells cultured without LDLs (not shown). However, it could not be excluded that the marked reduction of DNA synthesis and cell population size observed after PHA activation might be due, at least partly, to oxLDL-mediated apoptosis.

Oxidized LDLs induce several features of apoptosis in PHA-activated PBMC

To investigate whether oxLDLs induce an apoptotic process, chromatin fragmentation was evaluated by spectrofluorometry using DAPI fluorescent staining in PHA-activated PBMC after 3 day incubation with 200 μg apoB/ml native and oxLDLs (**Fig. 2A**). Between 24 and 72 h of culture, DNA fragmentation level increased quickly in populations exposed to oxLDLs, whereas the percentage of trypan blue-positive cells showed no significant modification (Fig. 2A, inset). These observations were confirmed by cytofluorometric analysis after nuclei staining by propidium iodide (PI), which clearly indicated that a significant amount of DNA was fragmented, with no significant increase in trypan blue percentage as compared to cells cultured with native LDLs (Fig. 2B and inset).

To study the relationship between LDLs, lipid peroxidation level and DNA fragmentation, experiments were carried out with LDLs oxidized during various UV radiation times. DAPI-evaluated chromatin fragmentation was positively correlated with radiation time, TBARS, and hydroperoxide levels (Fig. 2C).

As PBMC is a heterogeneous population containing monocytes, NK, B, and T lymphocytes, apoptosis was investigated in T-lymphocytes defined as $CD3^+$ cells. The expression of Apo2.7 antigen, considered as an early apoptosis marker, was examined by cytofluorometric analysis in CD3-positive cells from PHA-activated PBMC. The results showed that 200 μg apoB/ml oxLDLs significantly raised the $Apo2.7^+$ cell number among the $CD3^+$ population between 24 and 72 h culture, compared to the native LDL effect (Fig. 2D). In addition, Apo2.7 expression in $CD3^+$ cells increased after 48 h of culture in presence of LDLs (200 μg apoB/ml) oxidized during various times (Fig. 2D, inset).

Microscopic examination showed the following: the concomitant staining by SYTO and propidium iodide confirmed that in the presence of oxLDLs, an increased percentage of cells exhibited fragmented nuclei as compared to cells cultured with or without LDLs (**Fig. 3**, **G–I**). The nuclear features (fragmentation) of apoptosis were positively correlated with the UV radiation times (**Fig. 4A**). In addition, it is possible that apoptosis after oxLDL treatment was underestimated as microscopic analysis after MGG staining indicated that several cells showed nuclear shrinking and pyknotic features without clear fragmentation (Fig. 3C and 3F): more than 90% of 200 μg apoB/ml oxLDL-treated cells did not exhibit blastic morphology after 72 h culture and 33 ± 4% (means ± SD of three independent experiments) of cells underwent apoptosis (the cells became dense, shrinked, with packed, dense and pyknotic chromatin or karyorhexis) (Fig. 3 C, F, and I). Finally, the activated PBMC were double stained by Annexin V-FITC and PI to distinguish apoptotic cells from cells undergoing necrosis (Figs. 4B and 4C). After 48 h of culture, the percentage of Annexin V-positive cells increased from 10 ± 2% in the presence of 200 μg apoB/ml LDLs to 28 ±

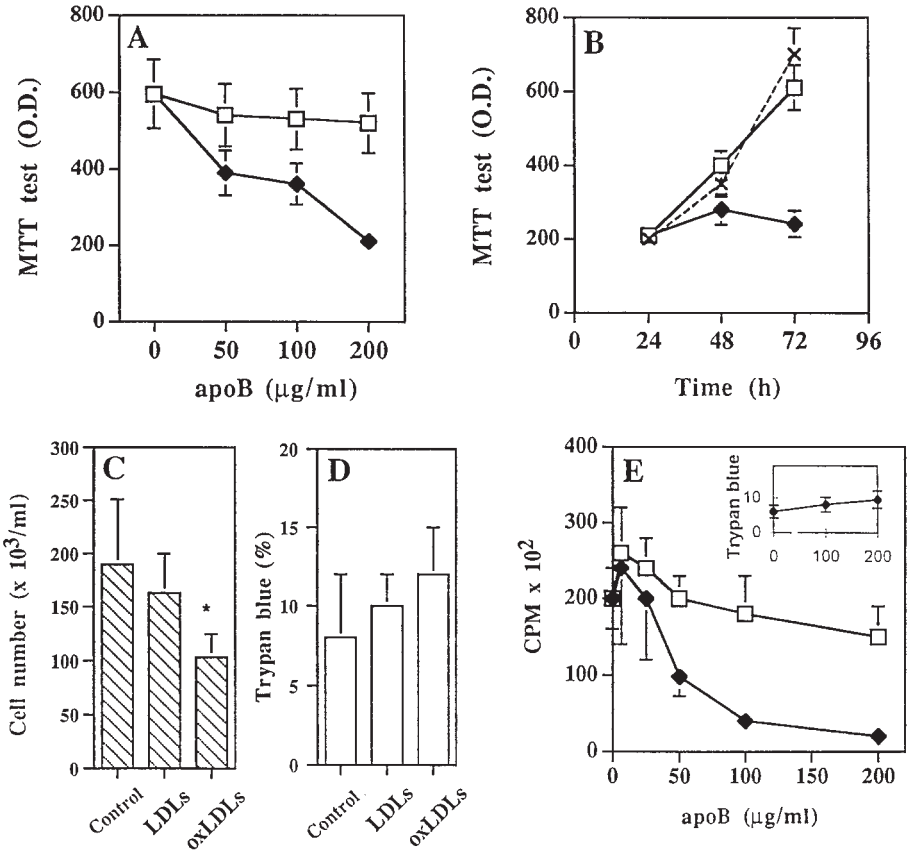

Fig. 1. OxLDL effects on cell growth and DNA synthesis in PHA-activated PBMC. PHA-activated PBMC were seeded in RPMI containing 2% LPDS. Results are means ± SD of 3 independent experiments for A, B, E and of 5 independent experiments for C and D. (A–D) Lipoprotein effect on cell growth. Cell proliferation was studied using MTT assay (A–B) and cell numeration in presence of trypan blue using a light microscope (C–D). The results of MTT assay are expressed as optical density (O.D.) obtained at $\lambda = 430$ nm. Dose-dependent effects (A) of LDLs (□) or oxLDLs (◆) were measured after 72 h culture. Time-dependent effects (B) were evaluated without (×) or with the addition of 200 μg apoB/ml LDLs (□) or oxLDLs (◆). PHA-activated PBMC were seeded (10^5/ml, 100 μl/well in triplicate) and the number of trypan blue negative cells (C) was measured after 72 h culture without (control) or with the addition of 200 μg apoB/ml native LDLs or oxLDLs; * $P < 0.05$ as compared to control cells. (E) Lipoprotein effect on DNA synthesis. Dose-dependent effect of LDLs (□) or oxLDLs (◆) were measured after 72 h incubation. The incorporated [^{3}H]thymidine amount is expressed as counts per minute (CPM). Inset shows necrosis index determined by the percentage of trypan blue-stained cells in the corresponding experiments.

3% in presence of oxLDLs (3 h UV radiation, means ± SD of three independent experiments). The induction of apoptosis increased as a function of LDL oxidation level (Fig. 4B). In addition, the analysis of the different stained cell populations showed that the majority of necrotic cells (PI positive cells) were probably undergoing secondary necrosis as cells that were PI-positive and Annexin V-negative were nearly absent.

Oxidized LDLs induce apoptosis in Jurkat T-cell line

We have shown that T-lymphocytes from PHA-activated PBMC undergo an increased rate of apoptosis in the presence of 200 μg/ml oxLDLs. In order to confirm oxLDL apoptotic effect on T-cells, we chose the Jurkat cell line as a model of proliferating T-lymphocytes.

DNA synthesis measured by [^{3}H]thymidine incorporation showed that 200 μg apoB/ml oxLDLs induced a strong inhibition of Jurkat proliferation after 24 h culture, accompanied by a low percentage of necrotic cells (**Fig. 5A**). For longer culture periods, up to 48 h, DNA synthesis was reduced over 60%, whereas in the presence of native LDLs, no significant decrease was observed. After 48 h culture, the percentage of dead cells evaluated by trypan blue exclusion test had increased from 8 ± 2% in cultures treated with native LDLs to 45 ± 2% in those treated with oxLDLs. The use of a dose range indicated a clear oxLDL effect on [^{3}H]thymidine incorporation after 24 h incubation with 200 μg apoB/ml (Fig. 5B).

Cell death assessed by trypan blue test indicated that oxLDLs are highly cytotoxic to Jurkat T-cell line after 48 h culture. However, it must be taken into account that trypan blue may detect post-apoptosis and primary necrosis as well. Experiments were carried out in order to determine whether trypan blue-positive cells had previously

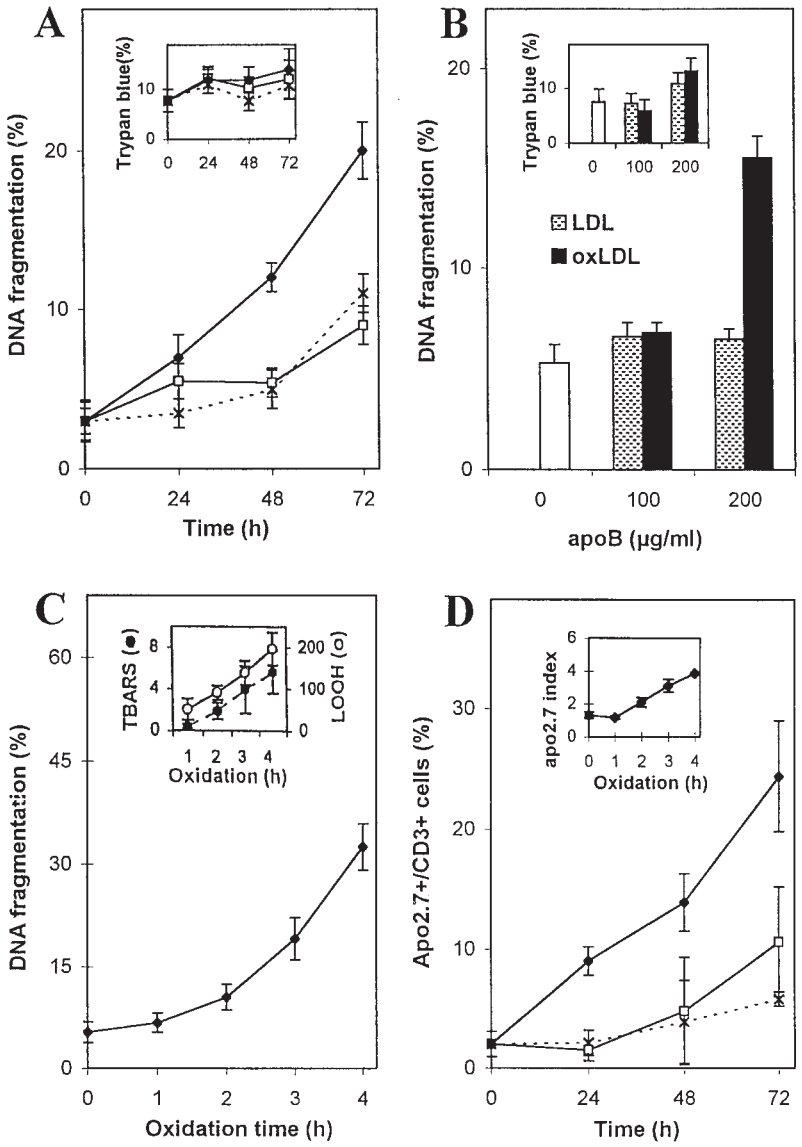

Fig. 2. OxLDLs induce DNA fragmentation and Apo2.7 antigen cell-surface expression in PHA-activated PBMC. PHA-activated PBMC (25×10^4/ml) were seeded in RPMI–2% LPDS. All results are means ± SD of 3 independent experiments. (A) Time-dependent effect of 200 μg apoB/ml lipoprotein: without (×) or with the addition of LDLs (□) or oxLDLs (◆). Results indicate the DNA fragmentation percentage evaluated by ultracentrifugation of chromatin fragments and DAPI staining. The inset shows the percentage of trypan blue positive cells observed during the experiments. (B) Dose-dependent effect after 48 h culture. Cells were permeabilized and nuclei were stained with PI. Histograms represent the DNA fragmentation level evaluated by cytofluorometric analysis. Trypan blue positive cell percentage is reported in the inset. (C) LDL oxidation level effect. LDLs were oxidized during increasing periods of time from 0 to 4 h (corresponding TBARS and hydroperoxide (LOOH) levels of oxLDLs are indicated in inset) and added to culture medium at a concentration of 200 μg apoB/ml. After 48 h culture, DNA fragmentation level was determined as in (A). (D) Effect on the Apo2.7$^+$ antigen expression. Cells were cultured during 24–72 h without (×) or with 200 μg apoB/ml LDLs (□) or oxLDLs (2.5 h UV radiation) (◆). Line graphs show the percentage of Apo2.7$^+$/CD3$^+$ cells determined by cytofluorometry and calculated within the whole lymphoid population. The inset represents the variation of Apo2.7$^+$/CD3$^+$ cells exposed for 48 h to 200 μg apoB/ml LDL oxidized during increasing periods of time (0–4 h). The results are expressed as an index of Apo2.7$^+$ expression modulation (in percentage of Apo2.7$^+$/CD3$^+$ cells) calculated as follows: (% in experiments with LDLs or oxLDLs − % of control without LDLs)/% of control without LDLs.

undergone apoptosis. Jurkat cells were exposed to 200 μg apoB/ml oxLDL during 24 h and compared to the lymphoblastoid B-cell line B.B. cultivated in the same conditions, as EBV infection is known to protect cells from apoptosis. Cells were analyzed by cytofluorometry after staining with PI and used as follows: *1*) in **Fig. 6A**, PI is added to non-permeabilized cells and detects loss of plasma membrane integrity in cells (i.e., necrosis) after 24 h culture; *2*) in figure 6B (as in Fig. 2B), PI is added to permeabilized cells to determine DNA fragmentation level. After 24 h treatment with 200 μg apoB/ml oxLDLs, a high level of DNA fragmentation was observed in Jurkat cells: 51 ± 3% versus 21 ± 2% in B.B. cells (Fig. 4 B). By contrast, only 20 ± 3% of Jurkat T-cells were PI$^+$ versus 56 ± 6% of lympho-

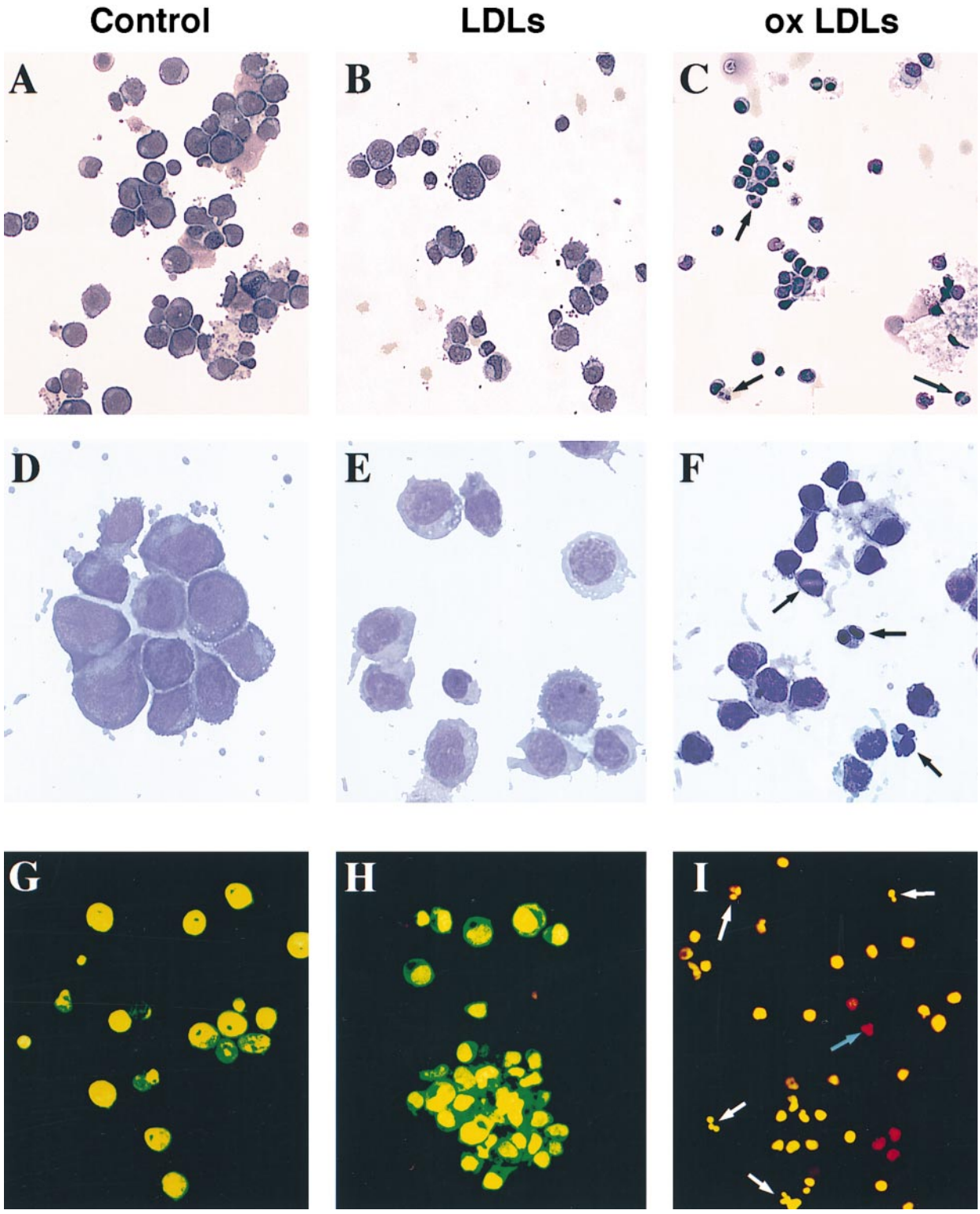

Fig. 3. Morphological analysis of apoptosis induced by oxLDLs in PHA-activated PBMC. PHA-activated PBMC (10^5/ml) were seeded in RPMI–2% LPDS without (Control) or with the addition of 200 μg apoB/ml native LDLs or oxLDLs. (A–F) After 72 h culture and MGG staining, cell morphological analysis was done using light microscopy. (C) (magnification: 400×) and (F) (1000×): In presence of oxLDLs several cells show characteristic features of apoptosis (cell shrinking, chromatin condensation, pyknotic nuclei and karyorhexis, for instance, see black arrows). (G–I) After 72 h culture cells were stained by vital fluorescent dyes, SYTO-13 and PI, then observed using a fluorescence microscope (400×). (I) Most cells show condensated nuclei; numerous nuclei are fragmented (for instance, see white arrows). One cell exhibits a clear post-apoptosis necrosis (fragmented red nucleus, blue arrow).

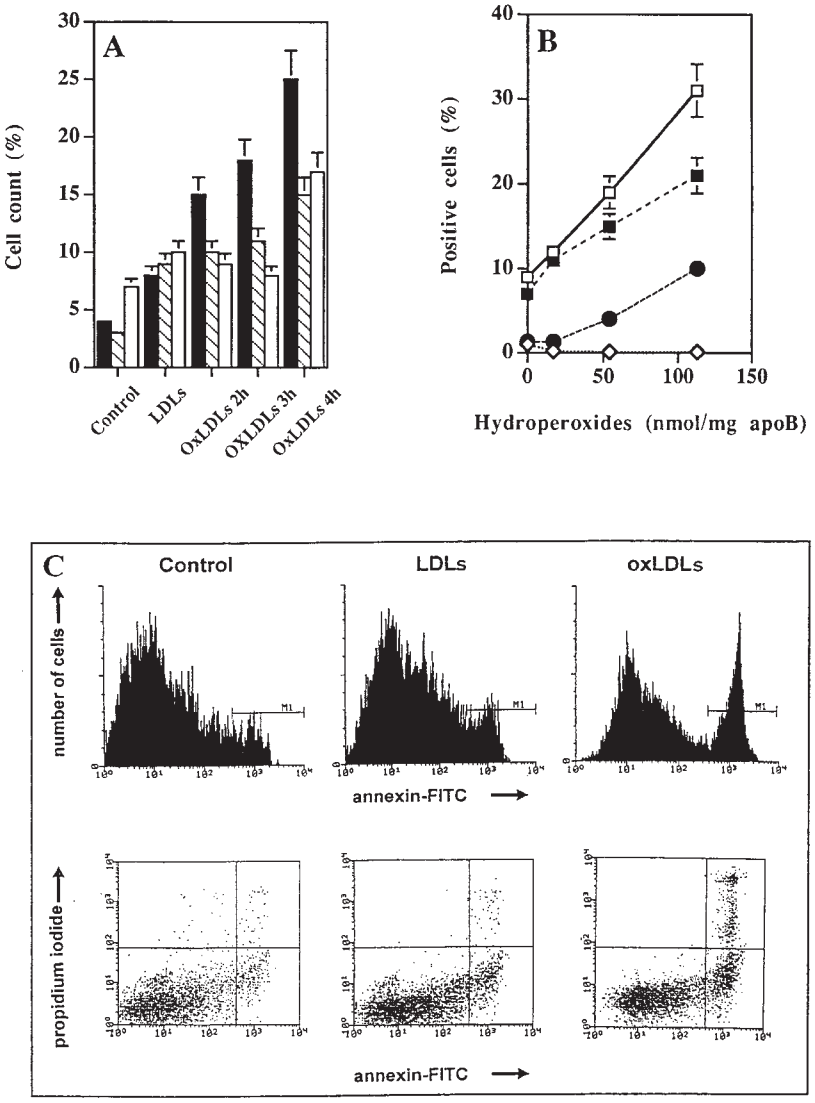

Fig. 4. Evaluation of necrosis and apoptosis by fluorescence microscopy and cytofluorometry in activated PBMC. PHA-activated PBMC were cultured in RPMI–2% LPDS. (A) The figure shows the percentage of SYTO-13 positive cells with fragmented yellow nuclei (black histogram, i.e., apoptosis), of PI positive cells (hatched histogram, i.e., necrosis), and of trypan blue positive cells (white) after 72 h culture in presence of 200 μg apoB/ml oxLDLs (2–4 h of UV radiation) or LDLs, or without LDLs (Control). Results are means ± SD of 3 independent experiments. (B and C) After 48 h of culture with or without 200 μg apoB/ml oxLDLs, the cells were stained by Annexin V-FITC and PI, then observed using a cytofluorometer. (B) Values are means ± SD from triplicate determinations. In this representative experiment the level of lipoprotein oxidation at time 0 was 17 nmol of hydroperoxides/mg apoB for LDLs, and 55 and 114 nmol/mg apoB for LDLs oxidized by 2 h and 3 h UV-radiation, respectively. Another separate experiment gave identical results. A third experiment was also performed only with LDLs oxidized by 3 h UV-radiation and gave identical results. Annexin negative/PI positive cells, i.e., primary necrosis (◇); Annexin positive/PI positive cells, i.e., secondary necrosis (●); Annexin positive/PI negative cells, i.e., apoptosis (■); Annexin positive cells (□). (C) Upper row: monoparametric histograms showing fluorescent intensity of cells stained by Annexin V-FITC. M1 gate contained Annexin positive cells. Lower row: biparametric analysis showing the viable cells (Annexin and PI negative) going across the apoptosis quadrant (lower right) to post-apoptotic secondary necrosis quadrant (upper right).

blastoid B-cells (Fig. 6A). In addition, DNA extracted from Jurkat T-cells after exposure to 200 μg apoB/ml oxLDLs during 24 h was analyzed by electrophoresis. The ethidium bromide gel showed the apoptotic ladder, characteristic of DNA internucleosomal cleavage (Fig. 6C). Last, light microscopy confirmed that a large number of Jurkat T-cells underwent apoptosis after 24 h culture in the presence of 200 μg apoB/ml oxLDLs. About 50% of Jurkat T-cells (versus 10% of B.B. cells) showed modifications such as cytoplasm and chromatin condensation, cell and nuclear fragmentation, which are compatible with cell death by apoptosis (Figs. 6 D–F).

DISCUSSION

The present results indicate that oxLDLs may trigger apoptosis in activated and proliferating T-lymphocytes: *i*) using fluorometric and morphologic assays, nuclear condensation and/or chromatin fragmentation are ob-

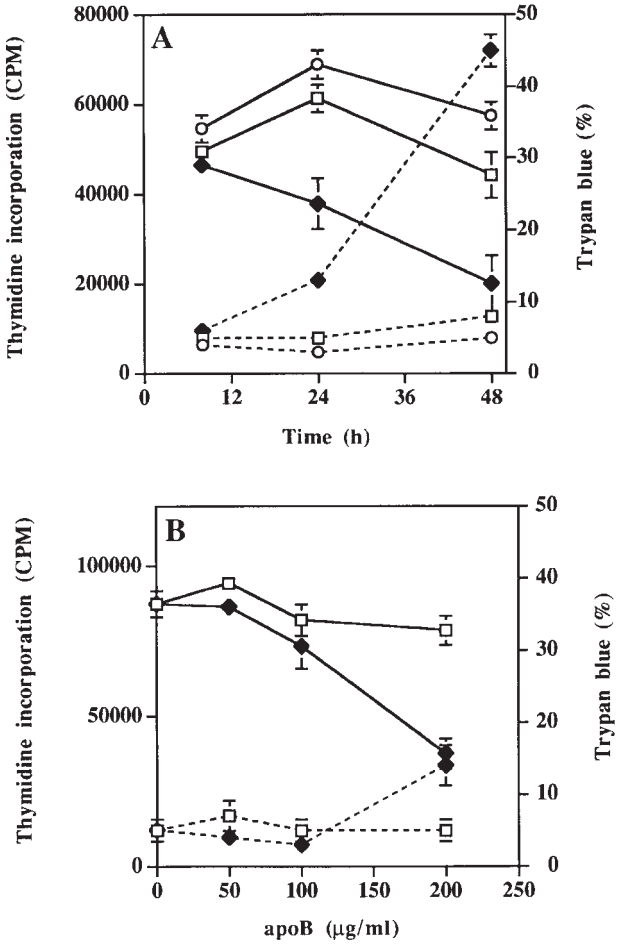

Fig. 5. OxLDL effects on the Jurkat T-cell line proliferation and viability. Jurkat cells were seeded in RPMI–2% LPDS (10^5/ml, 100 μl/well in triplicate cultures) and incubated for increasing periods from 0 to 48 h without LDLs (○), with native LDLs (□), and oxLDLs (◆), at doses up to 200 μg apoB/ml. All results are means ± SD of 4 independent experiments. (A) Time-dependent effect observed with 200 μg/ml apoB. Solid lines indicate DNA synthesis determined by measure of [^{3}H]thymidine incorporation and expressed as CPM. Dotted lines show the percentage of trypan blue-positive cells. (B) Dose-dependent effect observed after 24 h culture. Solid lines represent DNA synthesis evaluated and expressed as in (A). Dotted lines show the percentage of trypan blue-stained dead cells.

served in PBMC activated by PHA, a well-known polyclonal T-cell stimulator; *ii*) the expression of Apo2.7 antigen and Annexin V, two apoptosis markers, was increased in PHA-activated T-lymphocytes, whereas necrotic cells seemed to be mainly in post-apoptotic necrosis; *iii*) apoptosis was observed also in the Jurkat T-cell line. A monocyte-mediated induction of apoptosis in T lymphocytes cannot be excluded in PHA-activated PBMC incubated with oxLDLs. Indeed, numerous molecules produced by immune cells can induce or modulate apoptosis (35), e.g., tumor necrosis factor and interleukin-1, and the production of some of these may also be induced by oxLDL-treated monocytes (36, 37). Another possibility is the triggering of apoptosis by oxLDL-stimulated cytotoxic $CD8^+$ T-lymphocytes, which may kill autologous PHA-activated PBMC, as previously shown (38). However, the observation of apoptosis in the Jurkat T-cell line strongly suggests that oxLDLs may have a direct cytotoxic effect on activated and proliferating T lymphocytes. Similarly, it has been shown that oxLDLs can induce apoptosis directly in other cell types, such as endothelial cells (11), SMCs (10), and macrophages (12).

Under our experimental conditions, LDL oxidation results neither in a significant apoB alteration nor in a lysophosphatidylcholine accumulation and there is only a mild increase of hydroperoxides and oxysterols (25, 34). A recent report showed that only prolonged LDL exposure to UV radiation (over 20 h), causing apoB degradation, generates derivatives that induce apoptosis in SMCs and macrophages (10). Our present data obtained with mildly oxidized LDLs (UV radiation for 2–4 h) suggest that activated T-lymphocytes could be more susceptible to oxLDL-mediated cytotoxicity than the other cell types present in atherosclerotic lesions. This might explain the progressive T-lymphocyte disappearance from advanced plaques.

Concerning the component of oxLDLs that could induce apopotosis in our experimental system, several observations indicate that lipid peroxides such as oxysterols or 4-hydroxynonenal (4-HNE), a major lipid peroxidation product of oxLDLs, induce apoptosis in SMCs and in macrophages (39–42). Identical molecules could be involved in the apoptosis observed in activated T lymphocytes, as a positive correlation exists between TBARS and hydroperoxide levels and apoptosis rate. For instance, under our standard conditions, previous experiments demonstrated that oxLDLs contained about 6–10 nmol 4-HNE/mg apoB (43). Thus, 4-HNE can be one of the putative molecules involved in apoptosis induction in activated T lymphocytes. Particularly, 4-HNE is able to derivatize cell proteins which results in the modulation of cell function and induction of cytotoxicity (44). Recent reports using monocytic leukemia cells demonstrate that 7β-hydroxycholesterol, 7-ketocholesterol, and 25-hydroxycholesterol induced nuclear condensation and/or DNA fragmentation (41, 42). Finally, preliminary experiments in our laboratory indicate that 7β-hydroxycholesterol could induce apoptosis in Jurkat T cells (not shown).

In the present experiments, apoptosis prevailed over necrosis in activated PBMC as well as in the Jurkat T-cell line. The mechanism by which oxLDLs lead to apoptosis is still poorly understood. In contrast to results obtained with Jurkat cells, but in agreement with previous findings from our laboratory (45), the present results confirm that oxLDLs induce cell death in EBV-transformed B-cells mainly by necrosis. This might be due to intrinsic differences of susceptibility to oxLDL effects between B and T-lymphocytes, similar to the differential apoptosis susceptibility of T lymphocyte subsets recently described (46). Another possible explanation is the anti-apoptotic effect of EBV infection, such as the increased expression of Bcl-2 oncoprotein, which is known to be one of the key intracellular apoptosis regulators (47). Recent results in our laboratory suggest that a high level of Bcl-2 does not protect from oxLDL-induced cell death but rather shifts it toward

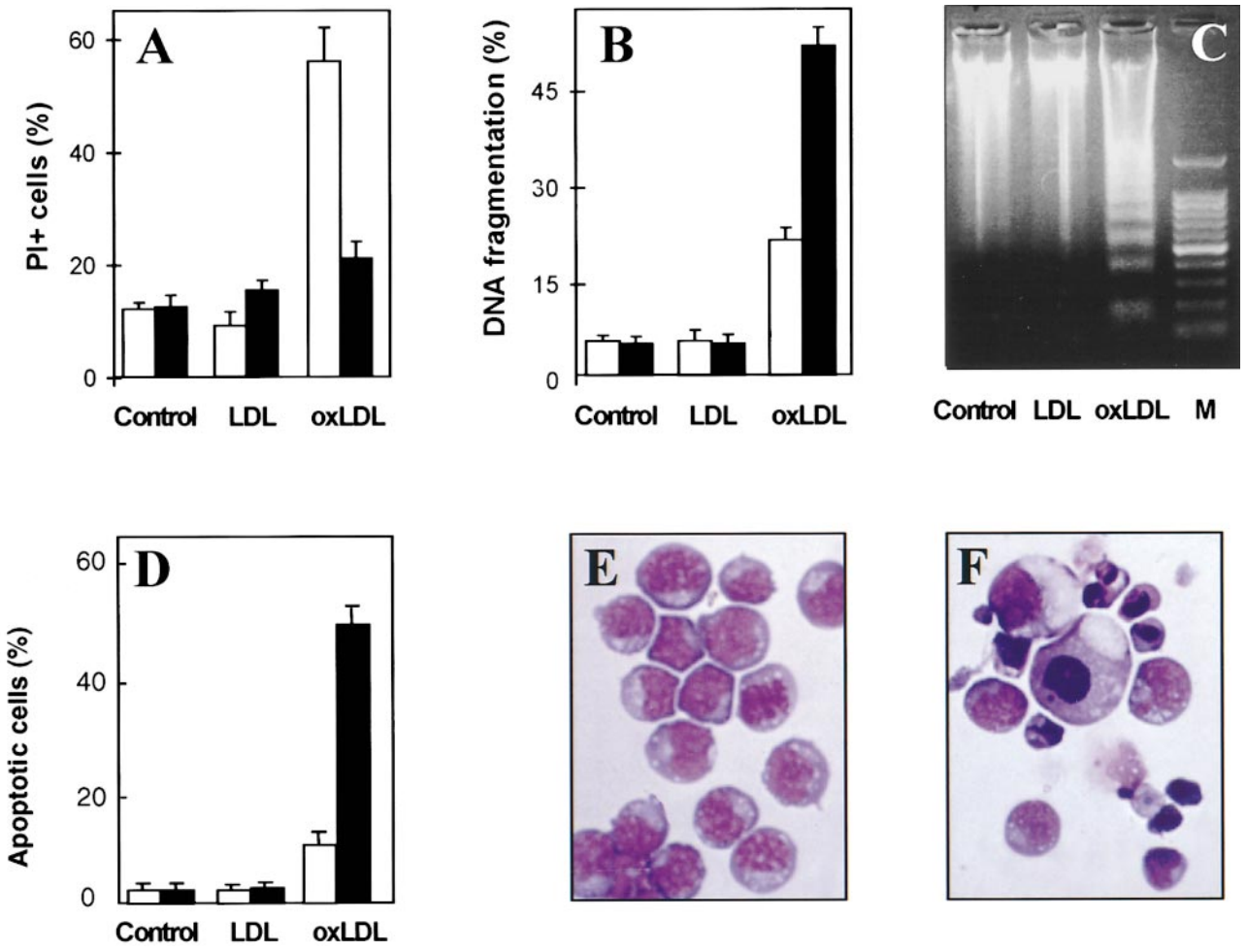

Fig. 6. OxLDL induce apoptosis in the Jurkat T-cell line and necrosis in the B.B. B-cell line. The B.B. lymphoblastoid B-cells (white histograms) and the Jurkat T-cells (black histograms) were seeded in RPMI–2% LPDS (10^5/ml, 100 μl/well in triplicate cultures) during 24 h with or without 200 μg apoB/ml LDLs and oxLDLs, then the cells were studied by various methods. Histograms express means ± SD of 3 independent experiments. (A) Plasma membrane integrity of oxLDL-treated cells: after 24 h culture cells were incubated with PI and studied by cytofluorometry. Results indicate the percentage of PI-permeant necrotic cells. (B) DNA fragmentation analysis: nuclei from permeabilized cells were stained with PI and DNA content was evaluated by cytofluorometry; the results represent the percentage of apoptotic DNA fragments. (C) Gel electrophoresis of DNA (under conditions described in Materials and Methods) of Jurkat T-cells treated for 24 h with 200 μg apoB/ml LDLs and oxLDLs. M indicates molecular weight markers. (D–F) Morphological analysis of oxLDL-induced cell death in the Jurkat and B.B. cell lines using light microscopy. Histograms show the percentage of morphological apoptotic cells after numeration. Photomicrographs show an example of results obtained with the Jurkat T-cell line: (E) untreated control cells, (F) oxLDL-treated cells undergoing apoptosis (nuclear and cytoplasmic condensation).

necrosis (48, and O. Meilhac, I. Escargueil-Blanc, J. C. Thiers, R. Salvayre, and A. Nègre-Salvayre, unpublished results). In activated PBMC, apoptosis seems to be neither a quick nor a massive phenomenon. This suggests that oxLDLs only amplify a normal process, observed in activated and proliferating populations and associated with the regulation of lymphoid cell expansion (49). Bcl-2 is constitutively present in resting T lymphocytes and is overexpressed in activated T cells. However, activation is known to induce apoptosis that is considered as a normal mechanism for maintaining homeostasis in lymphoid populations (49, 50). Numerous factors may be involved in this regulation. For instance, a drop of IL-2 may trigger apoptosis in activated T-lymphocytes in spite of a high level of Bcl-2 expression (46). Recent results demonstrate that mildly oxidized LDLs inhibited the IL2 secretion in PHA-activated lymphocytes (51).

Another possibility may be an alteration by oxLDLs of redox-sensitive apoptotic pathways in activated T-lymphocytes. Indeed, after activation, lymphocytes produce increased levels of reactive oxygen intermediates (ROI) which may serve as intracellular signaling molecules (52). When cellular antioxidant levels are insufficient (or when ROI levels are in excess), T-cell activation or exposure to extracellular source of ROI may induce oxidative stress that could be a physiological mediator of apoptosis (52). In a previous paper the level of TBARS was determined in lymphoid cells treated with UV-oxidized LDLs (25). After 48 h culture, the TBARS intracellular concentration was 26.5 nmol/mg cell protein in cells treated by 200 μg/ml

oxLDLs versus 0.4 nmol/mg cell protein in cells without LDLs and 2.4 nmol/mg cell protein in the presence of vitamin E, suggesting that oxLDLs strongly increased the level of molecules likely to modulate oxidative stress.

Apoptosis is an important physiological process in the maintenance of tissue homeostasis, complementing differentiation, migration, and cell proliferation. A disturbance of programmed cell death regulation is likely to lead to pathological conditions (9). Several observations have demonstrated that apoptosis is abundant in human atherosclerotic lesions in SMCs, macrophages, and T cells (27, 53, 54). Our present study suggests that oxLDLs may be responsible for T-lymphocyte apoptosis within atheromatous plaques. This cell death could be involved in an immune dysregulation and in an unappropriate local response of T lymphocytes.

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