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OXIDIZED LOW DENSITY LIPOPROTEINS INDUCE APOPTOSIS IN HUMAN LYMPHOCYTES: INVOLVEMENT OF MITOGEN-ACTIVATED PROTEIN KINASES

M. BUSTAMANTE[1,2], F. DÍAZ[1], M. MUÑOZ[1], H-J. GROSS[2], C.I. RIVAS[4], A. LLANCAQUEO[1], L. NÚÑEZ[1], L. CAMPOS[1], L. KIRSTEN[1], J. GRANDÓN[1], M. GONZÁLEZ[3], V. BARRA[4], J.C. VERA[4] AND M.G. BACHEM[2]

[1]Laboratory of Molecular Biology, Faculty of Medicine, Catholic University of Concepción, 2850 Alonso de Ribera Street, Concepción, Chile.
[2]Dept. Clinical Chemistry, University of Ulm, Ulm, Germany,
[3]Dept. Clinical Chemistry and Immunology, Faculty of Pharmacy and
[4]Dept. of Pathophysiology, Faculty of Biological Sciences, University of Concepción, University Town, Concepción, Chile.
Prof. Dr. Marcelo Bustamante Ph.D., Director Laboratory of Molecular Biology, Faculty of Medicine, Catholic University of Concepción, Alonso de Ribera 2850, Concepción, Chile. Tel.: ++56 41 2735435, FAX: ++56 41 2735435, Email: mbustamante@ucsc.cl

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Abstract – Oxidized low density lipoproteins (oxLDL), macrophages and T-lymphocytes are present in atherosclerotic lesions. We and others have shown that oxLDL is cytotoxic for macrophages, endothelial, smooth muscle and activated T-lymphocytes and induce apoptosis. Here we demonstrate that (i) oxidized LDL (oxLDL), oxidized VLDL (oxVLDL) and hydrogen peroxide (H_2O_2) induce apoptosis in human T-lymphocytes and (ii) mitogen-activated protein kinases are involved in this process. Apoptosis was monitored by immunofluorescence microscopy and flow cytometry for annexin V binding, Apo 2.7 expression, the TUNEL reaction and caspase 3 activity. In the presence of oxLDL (100 µg/ml), oxVLDL (50 µg/ml) and H_2O_2 (5 mM), the fraction of apoptotic cells increased within 6 hours to more than 70%. Preincubation of lymphocytes with the MAPKK inhibitor PD-98059 and the $p38^{MAPK}$ inhibitor SB-203580 almost completely abolished these effects. Furthermore, oxLDL and H_2O_2 but not native LDL strongly enhanced phosphorylation of JNK, $p38^{MAPK}$ and $p42/44^{MAPK}$. The results suggest that in the resting lymphocyte apoptosis triggered by oxidized lipoproteins and oxidative stress depends on the activation of $p44/42^{MAPK}$ and $p38^{MAPK}$ cascades.

Key words: apoptosis, lymphocytes, MAPK cascades activation, oxLDL, oxVLDL, oxidative stress

INTRODUCTION

Oxidative stress is implicated in the pathogenesis of atherosclerosis (1, 2). The clinical manifestations of atherosclerosis (such as coronary heart disease, stroke, heart infarct and peripheral occlusive arterial disease) are the first cause of morbidity and mortality among occidental people. A major event in atherogenesis is the excessive generation of reactive oxygen species (ROS, e.g. O^{2-}, $OH^{\cdot}$) which can promote lipid peroxidation of lipoproteins (3). Accumulating evidence suggests a role for immune competent cells in atherosclerosis. In fact, T lymphocytes precede monocytes in arterial intima infiltration and both can recognize oxidized LDL (oxLDL) (4). OxLDLs have been found in atherosclerotic lesions and is considered a key factor in atherogenesis (1), because they are taken up by macrophages via scavenger receptors (e.g. CD 36, LOX-1) and initiate foam cell formation (1, 5). OxLDLs also alter the cellular functions of endothelial cells, vascular smooth muscle cells, monocytes and macrophages (6, 7). Current evidence suggests that oxLDL might exert an inductive role in the atherosclerotic process by stimulating vascular smooth muscle cell

Abbreviations: oxLDL, oxidized low density lipoprotein; **oxVLDL**, oxidized very low density lipoprotein; **nLDL**, native low density lipoprotein; **nVLDL**, native very low density lipoprotein; **LOX**, Lectin like oxLDL receptor; **MAPKK**, mitogen activated protein kinase kinase; **MAPK**, mitogen activated protein kinase; **JNK**, Janus kinase; **ROS**, reactive oxygen species; **TUNEL**, Terminal transferase dUTP nick end labeling.

proliferation (8), endothelial cells dysfunction (7), synthesis of connective tissue components (9), and apoptosis of activated T-lymphocytes, macrophages and smooth muscle cells (9, 10, 11). The T-lymphocytes apoptosis seems to be decisive for the development of an inappropriate local immune response in the atherosclerotic plaque. Current research is focussing on the specific signal transduction cascades involved in the atherogenic process. Recently it was reported that the MAPK cascades could be responsible for some of the effects induced by oxLDL (12, 13, 14).

We show here that oxLDL, oxVLDL and hydrogen peroxide induce apoptosis in human lymphocytes within a few hours by triggering MAPK cascades, indicating that activation of T-lymphocytes is not the only way, required to gain susceptibility to the apoptotic process initiated by the oxidative stress. We suggest that in addition to oxidized lipoprotein-mediated apoptosis of activated T-lymphocytes, the corresponding effect occurring in lymphocytes also contributes to the pathogenesis of the atherosclerotic plaque.

MATERIALS AND METHODS

Materials

Antibodies and inhibitors

Monoclonal anti-APO 2.7 and streptavidin-PE were from Immunotech (Marseille, France), antibodies against p42/p44^{MAPK} and p38^{MAPK} respectively were from Calbiochem-Novabiochem, Schwalbach, Germany, monoclonal antibody anti phospho p44 (pThr^{202}/pTyr^{204}) and phospho p42 (pThr^{185}/pTyr^{187}) and anti phospho p38 MAPK (pThr^{180}/pTyr^{182}) were from Sigma (Saint Louis, MI, USA), anti SAPK/JNK and anti phospho-SAPK/JNK (Thr^{183}/Tyr^{185}) mAb from New England BioLabs, Frankfurt, Germany. The TUNEL-Assay (APO-Direct-Kit) was from Pharmingen (San Diego, CA, USA); annexin V-Biotin from Hölzel Diagnostika (Cologne, Germany); horseradish peroxidase (HRP)-conjugated anti-mouse IgG, HRP-conjugated goat-anti-rabbit IgG, fluorescein-conjugated streptavidin, biotinylated rabbit-anti-mouse IgG and HRP-conjugated streptavidin from DAKO (Hamburg, Germany); and tyramide signal amplification (TSA-Indirect) from NEN^{TM} Life Science Products (Boston, MA, USA). All other reagents were of analytical quality. MAPK inhibitors PD-98059 (I1) and SB-203580 (I2) were from Calbiochem-Novabiochem, Schwalbach, Germany.

Isolation and modification of lipoproteins

Lipoproteins were isolated from plasma of healthy donors using sequential ultracentrifugation and subsequently oxidized by exposure to air in the presence of 5 µM Cu^{2+} at 37ºC for 24 h (9, 15). Thereafter LDL was thoroughly dialyzed against degassed PBS pH 7,4 containing 4 mM EDTA at 4ºC to remove $CuSO_4$ and then dialyzed against PBS pH 7,4 without EDTA. Native LDL (nLDL), oxidized LDL (oxLDL), native VLDL (nVLDL) and oxidized VLDL (oxVLDL) were stored under Argon at 4ºC. The lipoproteins were used within 2 weeks of preparation (9). The oxidation of lipoproteins used for cultures was checked by the method described by Soukry, M. et al (1994) (15). In all experiments the protein concentration was determined using the BioRad protein assay (BioRad Laboratories, Munich, Germany), for nLDL and oxLDL was 100 µg/ml and for nVLDL and oxVLDL was 50 µg/ml, these values were obtained from a concentration response curve (data not shown). The approval to work with human blood was granted by the Ethical Commission of the Medicine Faculty of the Catholic University of Concepción.

Isolation and purification of lymphocytes

Mononuclear cells were isolated from buffy coats by density-gradient (Ficoll) centrifugation. Buffy coat were obtained from blood bank of German Red Cross (Ulm). Lymphocytes were purified by depletion of monocytes using CD14-coated magnetic beads and their purity was controlled by three colour flow cytometry, using anti-CD3, -CD4, -CD8. These lymphocytes were cultured for 6 hours at 37°C in the presence or in the absence of several concentrations (50 -100 µg/ml) of nLDL, oxLDL, nVLDL and oxVLDL, with and without 2 µM of PD-98059 (an inhibitor of mitogen-activated protein kinase kinase (MAPKK), or 2 µM of SB-203580 (an inhibitor of p38^{MAPK}). Lymphocytes treated with 5 mM H_2O_2 for 6 h at 37°C were used as positive controls for oxidative stress-induced apoptosis. For the mononuclear cells isolation from buffy coats was not necessary an Ethical Commission approval.

Annexin V-binding

The annexin V-binding assay was performed identically for immunofluorescence microscopy and flow cytometry. After the 6 h treatment, the cells were washed with PBS and fixed for 1 h in PBS-buffered 1% formaldehyde, and non-specific binding was blocked with TBS-buffer (10 mM Tris, 150 mM NaCl, pH 7.4) containing 0,5 % bovine albumin (TNB-buffer) for 45 min at room temperature (RT). Afterwards, the cells were incubated with biotinylated annexin V (diluted 1/30 in TBS with 2 mM $CaCl_2$) for 1 h at RT, washed (3x) with TBS containing 0.05% Tween 20 (TNT) and 2 mM $CaCl_2$, and incubated with streptavidin-phycoerythrin (1/100 in TNB with 2 mM $CaCl_2$) for 1 h at RT. After washing with TNT-buffer with $CaCl_2$, nuclei were counterstained by staining with 5 µM bisbenzimide for 30 min at RT.

APO 2.7-Expression

After the 6 h treatment, the cells were washed in PBS and fixed in –20°C acetone. Non-specific binding was blocked with 0,5% bovine serum albumin (BSA) in PBS for 45 min at RT and subsequently the 1st antibody (Anti-APO2.7) diluted 1/20 in PBS/albumin was added and incubated for 2 h at RT. After washing with PBS, biotinylated anti-mouse IgG diluted 1/50 was added for 1 h, followed by 1 h incubation with streptavidin-peroxidase (1/50). After washing, tyramide reagent (1/40) was added for 15 min, and after three more washing steps streptavidin-phycoerythrin (diluted 1/100) was added for another hour. Nuclei were counterstained with 5 µM bisbenzimide for 30 min at RT. APO 2.7-positive and negative cells were visualized and photographed using an epifluorescence microscope (C. Zeiss, Oberkochen, Germany) or analyzed by flow cytometry (EPICS XL Coulter Immunotech, Hamburg, Germany) using anti-CD3 as a T-lymphocyte

specific surface marker.

TUNEL-Reaction

The TUNEL-reaction was performed identically for immunofluorescence microscopy or flow cytometry. After 6 h treatment, the cells were washed once with PBS pH 7.4 and fixed for 1 h in buffered (pH 7.4) 4% formaldehyde. After washing (3x), cell membranes were permeabilized by incubation with 70% (v/v) ethanol in PBS for 2 h. Staining was performed by incubating the cells overnight in the dark at 22°C with a solution consisting of 10 µl terminal deoxynucleotidyl transferase (TdT)-reagent, 0.75 µl TdT-enzyme, 8 µl FITC deoxy-uridin-triphosphate (dUTP) and 32 µl distilled water. The next day, the cells were incubated twice for 30 min with 50 µl of rinse-buffer and then incubated with 50 µl propidium iodide/RNAse solution in the dark for 30 min at RT. Finally, the cells were washed 3x with PBS and analyzed by fluorescence microscopy or flow cytometry.

Caspase assay

Caspase 3 activity measurement was carried out as described by Hug et al. (16) using rhodamine 110 (R)-labeled peptide (D2R). Briefly, after apoptosis induction cells were incubated with D2R 50 mM and dithiotreitol 10 mM for 10 min at 37 ^{o}C and used directly for FACS measurement.

Immunoblot

Cells were homogenized in lysis-buffer (10 mM Tris-HCl, pH 7.4, 4 mM EDTA, 1 mM PMSF, 40 mM NaF, 20 mM benzamidin and 1% (v/v) Triton X-100) after 1 h incubation at RT and the cell extract was centrifuged at 5000 x *g* for 10 min. The supernatant was transferred to a fresh tube and stored at –80°C until used. Protein concentration was determined using the BioRad protein assay.

For immunblotting, sample aliquots equivalent to 10 µg protein were boiled in 20 µl sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 2 mM dithiotreitol), fractionated in 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes using a semi-dry blot system. Phosphorylation of p42/44^{MAPK} was detected using the monoclonal antibody anti-phospho p42^{MAPK} (pThr^{202}/pTyr^{204}) and anti-phospho p44^{MAPK} (pThr^{185}/pTyr^{187}) diluted 1/1000 in PBS. Phosphorylation of JNK was detected using the phospho-SAPK/JNK (Thr183/Tyr185) mouse mAb. The load control was verify using anti-JNK antibody. Phosphorylation of p38^{MAPK} was detected using the anti-phospho p38^{MAPK} (pThr180/pTyr182) diluted 1/1000 in PBS. To verify the load control, antibodies against p42/p44^{MAPK} and p38^{MAPK} respectively were used. Nitrocellulose membranes were incubated for 1 h with the first antibody, washed extensively and incubated for 4 h with the secondary biotinylated antibody (anti-mouse IgG or anti-rabbit IgG 1/500 in PBS). After extensive washing, streptavidin-HRP was added at RT for 1 h and developed using the ECL system (Cell Signaling Technology, Danvers, MA, USA).

Statistical analysis

All quantitative measurements were done at least in triplicate. The results represent the mean $\pm$ SD of at least three independent experiments, the $p < 0,05$ was considered as statistically significant. Each experimental condition was investigated in three different culture wells. The T-test was employed to compare the different groups and the software used was Origin 6.0.

RESULTS

OxLDL-induced apoptosis of lymphocytes and the effect of MAPK-cascade inhibitors

To study whether oxLDLs induce apoptosis, lymphocytes from healthy donors were pre-incubated for 2 h with or without PD-98059 (a p42/p44 MAPK-cascade inhibitor) or SB-203580 (a p38 MAPK inhibitor) and then were cultured for 6 h in the presence of nLDL and oxLDL and the occurrence of apoptosis was analyzed by fluorescence microscopy. Figure 1 shows the results of the analysis of Annexin V-binding (panels A-E), Apo 2.7 expression (panels F-J) and the TUNEL reaction (panels K-O). Control cells incubated for 6 hours in the absence (Fig. 1A, 1F, 1K) or in the presence (Fig. 1B, 1G, 1L) of 100 µg/ml of nLDL showed very low to undetectable levels of apoptosis as assessed by Annexin-V binding, Apo 2.7 expression and the TUNEL reaction. A marked increase in the apoptotic cell fraction (Fig. 1C, 1H, 1M) was induced in human resting lymphocytes from healthy donors after incubation with 100 µg/ml oxLDL for 6 h. The increased apoptosis observed in lymphocytes treated with oxLDL was absent in cells pre-incubated with 2 µM of PD-98059 (Fig. 1D, 1I, 1N) Control cells pre-incubated with PD-98059 alone showed no staining for the apoptotic markers (data not shown). Furthermore, a very low to undetectable apoptosis was also observed in oxLDL-treated cells that were pre-incubated with 2 µM of SB-203580 (Fig. 1E, 1J, 1O).

OxVLDL-induced apoptosis of lymphocytes and effects of MAPK-cascade inhibitors

Next, we studied the capacity of oxVLDL to induce apoptosis in resting lymphocytes. For this, the cells pre-incubated for 2 h with or without PD-98059 or SB-203580 were cultured for 6 hours in the presence of 50 µg/ml nVLDL or oxVLDL, followed by analysis of annexin-V binding and Apo 2.7 expression. As a positive control for apoptosis induction by oxidative stress, the effect of 5 mM H_2O_2 on lymphocytes was also studied. Figure 2 shows the results of the analysis of annexin V-binding (panels A-H) and Apo 2.7 expression (panels I-P). Control cells incubated alone for 6 hours (Fig. 2A, 2I), or in the presence (Fig. 2B, 2J) of 50 µg/ml of nVLDL showed very low to undetectable levels of apoptosis. Treatment with 50 µg/ml oxVLDL or 5 mM H_2O_2 caused a strong induction of

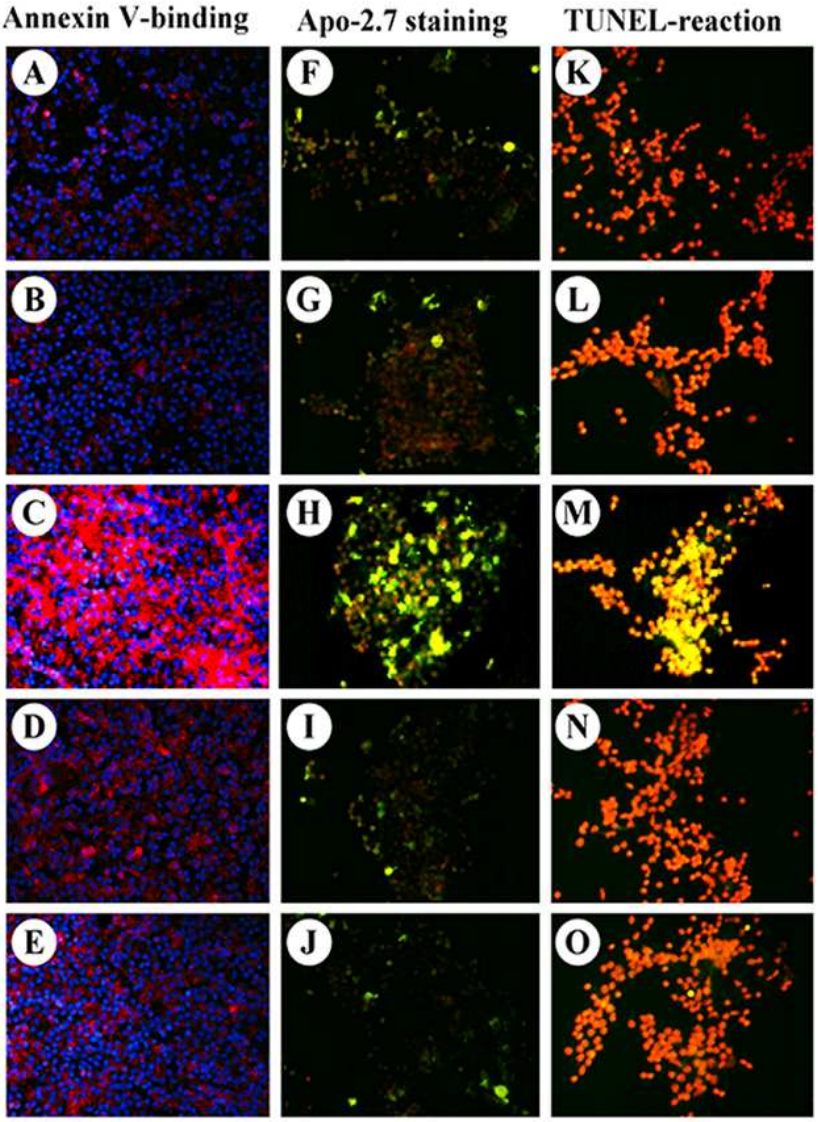

Figure 1. Immunofluorescence microscopy of annexin-V binding, Apo 2.7 expression and TUNEL reaction in oxLDL-treated cells. Human T-lymphocytes were cultured for 6 h with nLDL or oxLDL after a 2 h pre-incubation with or without of the MAPK-cascade inhibitors PD-98059 and SB-203580. Panels A-E: annexin V-binding assay; panels F-J: Apo 2.7 expression; panels K-O: TUNEL reaction. A, F, K: control cells. B, G, L: 100 µg/ml nLDL-treated cells. C, H, M: 100 µg/ml oxLDL-treated cells. D, I, N: 100 µg/ml oxLDL plus 2 µM PD-98059-treated cells. E, J, O: 100 µg/ml oxLDL plus 2µM SB-203580-treated cells.

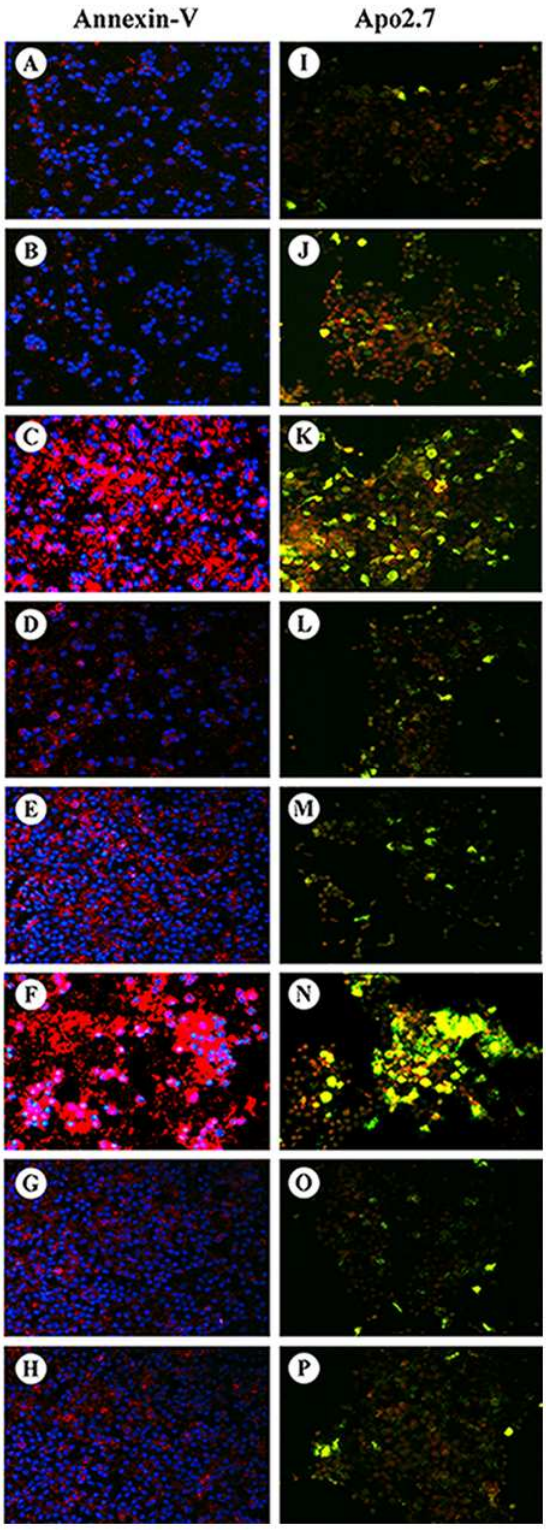

Figure 2. Immunofluorescence microscopy of annexin-V binding and Apo 2.7 expression in oxVLDL and H_2O_2-treated cells. Human T-lymphocytes were incubated for 6 h with nVLDL, oxVLDL or H_2O_2 after a 2 h pre-incubation with or without of the MAPK-cascade inhibitors PD-98059 and SB-203580. Panels A- H: annexin V-binding assay; panels I-P: Apo 2.7 expression. A, I: control cells. B, J: 50 µg/ml nVLDL-treated cells. C, K: 50 µg/ml VLDL-treated cells. D, L: 50 µg/ml oxVLDL plus 2 µM PD-98059-treated cells. E, M: 50 µg/ml oxVLDL plus 2µM SB-203580-treated cells. F, N: 5mM H_2O_2-treated cells. G, O: 5mM H_2O_2 plus 2µM PD-98059-treated cells. H, P: 5mM H_2O_2 plus 2µM SB-203580-treated cells.

apoptosis as revealed by a considerable increase in the number of cells positive for annexin-V binding or Apo 2.7 expression (Fig. 2C, 2K, 2F, 2N). Pre-incubation with both, PD-98059 (Fig. 2D, 2L, 2G, 2O) and SB-203580 (Fig 2E, 2M, 2H, 2P) blocked the apoptotic effect induced by oxVLDL or H_2O_2 .

Quantitation of apoptosis by flow cytometry

For exact quantization of the effect of oxLDLs on induction of apoptosis and the effects of the MAPK cascade inhibitors, samples processed for annexin-V binding, Apo 2.7 expression and the TUNEL reaction were analyzed by flow cytometry (Fig. 3). This analysis revealed that untreated lymphocytes (control) showed a very low level of annexin-V binding, with less that 5% of positive cells (Fig. 3A, black bar 1). Treatment of the lymphocytes with nVLDL or nLDL caused a minor increase in the number of cells positive for annexin V that not exceeded 20% of the cells (Fig. 3A, black bars 2, 3). In contrast, treatment of lymphocytes with oxVLDL and oxLDL caused a major increase in the number of annexin V positive cells, to a level higher than 90% of the cells (Fig. 3A, bars 4, 5). This effect was similar for both types of oxidized lipoproteins. The inhibitors PD-98059 (Fig. 3A, bars 6, 7) and SB-203580 (Fig. 3A, bars 8, 9) decreased the number of apoptotic cells to less than 40% of the population in the presence of oxVLDL or oxLDL. In both cases, the inhibitor PD-98059 was more efficient than SB-203580 in blocking the effect of the oxidized lipoproteins. Thus, PD-98059 decreased the fraction of annexin V positive cells to 25% in lymphocytes treated with oxVLDL, compared with a 37% of positive cells in samples treated with oxLDL and SB-203580. Control studies showed that the staining level obtained in presence of the inhibitors alone was not significantly different from that measured in presence of the native lipoproteins (Fig. 3A, bars 10, 11). Treatment of the lymphocytes with 5 mM H_2O_2 increased the percentage of cells positive for annexin V binding to more than 90% of the cell population (Fig. 2A, bar 12), but the pre-incubation with inhibitors PD-98059 and SB-203580, decreased the positive cells for annexin V-binding to less than 40% (Fig. 3A, bars 13-14).

Similar results were observed examining Apo2.7 expression (Fig. 3B), with the only difference that control, untreated lymphocytes showed a high level of Apo2.7 expression, with approximately 50% of positive cells (Fig 3B, black bar 1). Treatment of the lymphocytes with nVLDL or nLDL did not affect the number of cells positive for Apo2.7 expression (40-45% of the cell population remained positive; Fig. 3B, black bars 2, 3). In contrast, treatment of the lymphocytes with oxVLDL and oxLDL increased the fraction of Apo2.7 positive cells to about 80% of the cell population (Fig. 3B, bars 4, 5). In the presence of the inhibitors PD-98059 and SB-203580, the percentage of positive cells was decreased to about 40-45% (Fig. 3B, bars 6-9). Control studies showed that the inhibitors alone caused a level of staining for Apo2.7 similar to that observed in control, untreated cells (Fig. 3B, bars 10, 11). On the other hand, treatment of the lymphocytes with H_2O_2 increased the percentage of Apo2.7 positive cells to nearly 100% (Fig. 3B, bar 12), while in the presence of the inhibitors PD-98059 and SB-203580, the fraction of Apo2.7-positive cells decreased to 40% (Fig. 3B, bars 13, 14).

The results described above were confirmed applying the TUNEL assay (Fig. 3C). Control, untreated lymphocytes showed a very low level of staining, with about 10% of the cells positive by flow cytometry (Fig. 3C, black bar 1). Treatment of the lymphocytes with nVLDL or nLDL failed to affect the number of cells positive for the TUNEL reaction (Fig. 3C, black bars 2, 3), which never exceeded 10% of the cell population. In contrast, treatment of the lymphocytes with oxVLDL and oxLDL caused a considerable increase in the number of cells positive for the TUNEL reaction to a level of 70% (Fig. 3C, bars 4, 5). This effect was similar for both types of oxidized lipoproteins. The inhibitors PD-98059 and SB-203580 decreased the fraction of positive cells to 10% in the presence of oxVLDL or oxLDL (Fig. 3C, bars 6-9). Control studies revealed that the inhibitors alone caused a level of staining similar to that observed with the untreated cells (Fig. 3C, bars 10, 11). Treatment of the lymphocytes with H_2O_2 increased the percentage of cells positive for the TUNEL reaction to more than 90% (Fig. 3C, bar 12), and the inhibitors PD-98059 and SB-203580 decreased the percentage of positive cells to a basal level of 10% (Fig. 3C, bars 13, 14). The activity of caspase 3 was measured by flow cytometry and the results show that caspase 3 is activated when the T-lymphocytes are incubated with oxLDL or H_2O_2. (Fig. 3D, bars 3 and 4 respectively)

Western blot analysis of MAPK phosphorylation

Analysis of $p42/44^{MAPK}$ phosphorylation revealed a basal level of a phosphorylated product in control lymphocytes (Fig. 4A, lane 1) that was not increased by treatment with nLDL (Fig. 4A, lanes 2). OxLDL and H_2O_2 induced a major increase in the amount of two phosphorylated products as showed by a typical double band, indicating the phosphorylation of both kinases (Fig. 4A, lane 3 and 4 respectively). The MAPKK inhibitor, PD-98059 (I1) abolished the effects induced by the lipoproteins and H_2O_2 (Fig. 4A, lane 5, 6 and 7 respectively). The phosphorylation of JNK was also increased by treatment with oxLDL and H_2O_2. (Fig. 4B, lanes 3 and 4). Similar results respect to figure 4 were found when analyzing the phosphorylation of $p38^{MAPK}$; oxLDL and H_2O_2 induced a major increase in the amount of phosphorylated product (Fig. 4C, lanes 3 and 4), this effect was also abolished by the SB 203580 p38MAPK inhibitor (I2) (Fig. 4C, lanes 5, 6 and 7).

DISCUSSION

Since Brown and Goldstein (17) showed that macrophages were able to internalize modified LDL, and that this event leads to foam cell formation, a new research area emerged in vascular pathology investigating the effect of modified LDL on immune competent cells. Thus, T- and B-lymphocytes have been shown to be present in the atheroma plaque (4, 18). The presence of B-cells in the atherosclerotic lesions is important for the production of antibodies against modified LDL (18,19). On the other hand, T- lymphocytes purified from the atheroma lesions are able to bind oxidized LDL (4). There is convincing evidence that the atherosclerotic process is primarily triggered by biochemical mechanisms involving immune competent cells and that oxidatively modified lipoprotein is a key factor in the pathogenesis of atherosclerosis (1, 5, 20).

T-lymphocytes and monocytes are recruited early from peripheral blood to the atherosclerotic lesion (5, 21). Whereas the role of oxidized lipoproteins in the differentiation of monocytes into macrophages, foam-cell formation (1, 5) and apoptosis of macrophages and smooth muscle cells (2, 6, 9) is well established, only limited data are available on the molecular effects of oxidized lipoproteins on T-lymphocytes (4, 5, 11). OxLDL is a chemoattractant for T-lymphocytes, but induces apoptosis in the absence of monocytes (22). It has also been reported that the activation of the T-lymphocytes is initiated by plaque-derived antigen (one of them could be oxLDL) and the intensity of the activation depends on the degree of LDL oxidation (22, 23).

OxLDL in high concentration induces oxidative stress and apoptosis in macrophages, phytohemagglutinin-activated T-Lymphocytes, endothelial, smooth muscle cells and human fibroblast (2, 6, 9, 24). On the other hand, in low concentrations it induces cell proliferation (9). In the present study, we show that oxidized lipoproteins also induce apoptosis in resting T-lymphocytes and MAP kinases are involved in the apoptotic pathway. The functional integrity of T-lymphocytes seems to be important for the control of the atherosclerotic process, as a decreased T-lymphocytes count caused by immnunodeficiencies like AIDS or experimental depletion by drugs accelerates the atherosclerotic process (25, 26). In smooth muscle and endothelial cells oxidized LDL stimulates several transcription factors and induces proapoptotic alterations such as down regulation of Bcl-2 family proteins via the mitogen-activated protein kinase and Jun kinase pathways (14, 27). Alcouffe et al. (28) have also reported that the MAPK cascades could be involved in the apoptotic process of T-lymphocytes induced by oxLDL. T-lymphocyte apoptosis induced by oxidative stress mediated by oxidized lipoproteins may be another crucial event in the pathogenesis of atherosclerosis. The lipid peroxides found in oxidized lipoproteins, such as oxysterols or 4-hydroxynonenal (4-HNE), have been identified as inducers of apoptosis in human fibroblast, smooth muscle cells and macrophages (24, 27), and are presumably also responsible for apoptosis induction in activated T-lymphocytes. This is also supported by our findings revealing that lower concentrations of oxVLDL produce the same effect than oxLDL, an observation indicating that the LDL receptor is not involved and suggesting that the responsible of the effects of oxVLDL would be the oxidized fatty acids which are more concentrated in oxVLDL (9). Although it has been described that T-lymphocytes activated by treatment with minimally oxidized LDL are more sensitive than other cells to the oxLDL-mediated cytotoxicity at the atheroma plaque (12), the mechanism by which oxidized LDL can trigger apoptosis in lymphocytes has not yet been

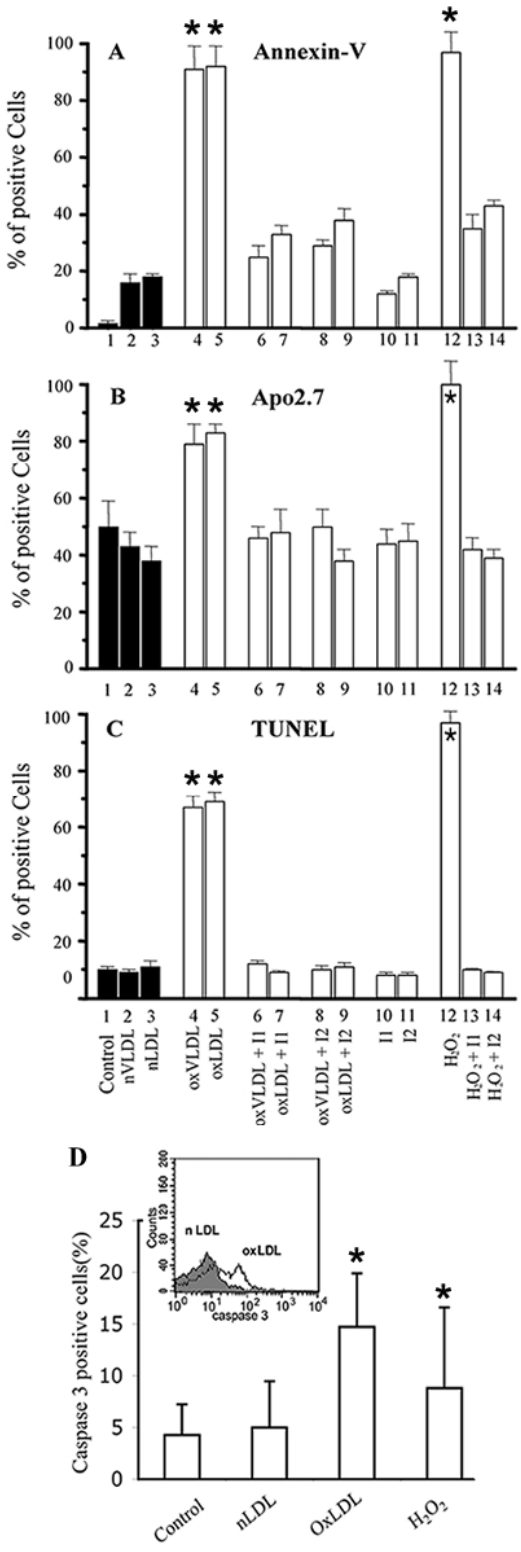

Figure 3. Quantitative analysis by flow cytometry of annexin-V binding, Apo 2,7 expression, TUNEL reaction and caspase 3 activity. A) Annexin V- binding assay, B) Apo-2.7 expression, C) TUNEL reaction (specific treatment conditions correspond to those described in the legend of Figure 1) and D) Measurement of caspase 3 activity by flow cytometry. I1 and I2 correspond to PD-98059 and SB-203580 respectively. Insert in Fig 3D correspond a typical cytometric analysis of caspase 3 activity. Asterisks (*) indicate that the values are significant respect to the control ($p < 0,05$).

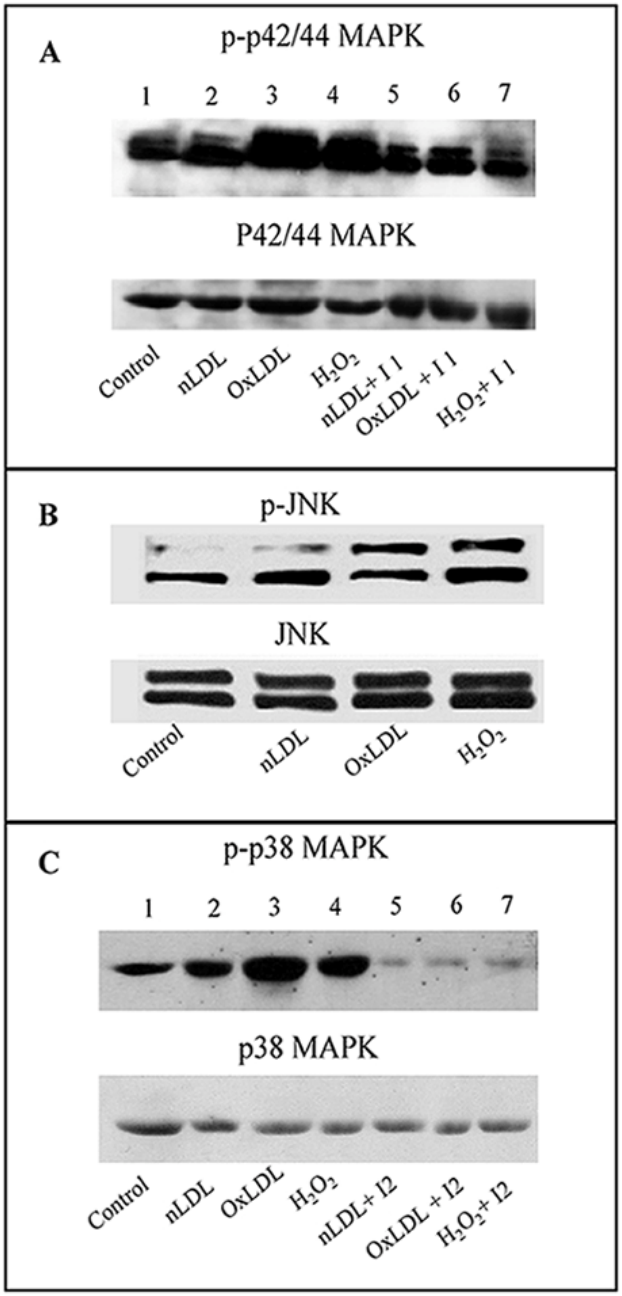

Figure 4. Western blot of p42/44, JNK and p38MAPK tyrosine phosphorylation. A) $p42/44^{MAPK}$ phosphorylation. B) JNK phosphorylation. C) $p38^{MAPK}$ phosphorylation. The numbers at the top correspond to those indicated in the text. Lipoproteins and inhibitors were added as described in the legend of Figures 1 and 2. The numbers at the bottom of each panel correspond to those indicated in the text. The used fractions for western blot analysis were prepared as described in Methods. The figures represent the results of 3 independent experiments.

elucidated, but there are evidence that some components found in oxidized lipoproteins like oxysterols, lysophosphatidylcholine (29) and oxidized cardiolipin (30) may up-regulate both expression and synthesis of monocyte chemotactic protein 1 (MCP-1) and transforming growth factor beta1 (TGFbeta1) (31), these mediators could stimulate their respective signal transduction cascade finally inducing inflammatory mechanism. In macrophages, it was also reported that oxLDL could activate the p42/44 MAP kinases by stimulation of a G protein-binded scavenger receptor and a calcium-dependent protein kinase C (13). It has been suggested that oxLDL may act by enhancing the physiological apoptotic process occurring in activated peripheral blood mononuclear cells (PBMC) (2) or it may act as an antigen that activates T-lymphocytes in the plaque (23). These effects may also occur at the atherosclerotic plaque, where the recruitment of resting T-lymphocytes from peripheral blood is one of the earliest pathogenic events.

In mixed cultures of mononuclear cells, macrophages produce mediators, which might induce apoptosis of T-lymphocytes, such as TNF-α and IL-1, and their release from macrophages is increased by oxLDL (32). Conversely, oxLDL-stimulated CD8+ T-lymphocytes induce apoptosis of PHA-activated mononuclear cells (11) suggesting a cross-stimulation between monocytes and lymphocytes.

Defining the signaling events induced or enhanced by oxidized lipoproteins in T-lymphocytes leading to apoptosis is thus an important prerequisite to gain further insight into the pathogenic process of atherosclerosis and to find novel therapeutic targets. The key finding of the present study was the observation that oxLDL and oxVLDL induced apoptosis in resting T-lymphocytes within a few hours. Occurrence of apoptosis was demonstrated by several independent methods: annexin-V binding, Apo 2-7 expression, the TUNEL-reaction and activation of caspase 3. On the other hand, both oxLDL and oxVLDL strongly enhanced the phosphorylation of JNK and both MAP Kinases and the apoptosis induction was efficiently blocked by two specific inhibitors of MAPK signal transduction pathways, PD-98059 and SB-203580 respectively. Since PD-98059 is an inhibitor of the MAPKK, and SB-203580 is an inhibitor of the $p38^{MAPK}$, we conclude that activation of JNK and both $p44/42^{MAPK}$ and $p38^{MAPK}$ cascades are crucial for the induction of resting human T-lymphocyte apoptosis by oxLDL and oxVLDL. It has been shown that apoptotic and necrotic death markers can concomitantly be present in the same cell after cerebral ischemia, indicating that more than death program may be activated at the same time (33). Moreover, this study indicates that activation of T-lymphocytes is not the only prerequisite to gain susceptibility to the apoptotic process initiated by oxidative stress, an effect that may contribute to the T-lymphocyte depletion found in atherosclerotic lesions.

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