Oxidized LDL triggers phosphatidylserine exposure in human monocyte cell lines by both caspase-dependent and -independent mechanisms

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Abstract Monocytic cell lines have been extensively used to characterize and model various features of the atherogenic process. We found striking differences in the apoptotic pathways of U937 cells and THP-1 cells exposed to copper-oxidized LDL. While phosphatidylserine exposure occurred in both lines, caspase activation was only apparent in the THP-1 cells. OxLDL caused caspase activity to decrease below that seen in untreated U937 cells, and this corresponded with a loss in intracellular thiols. In conclusion, exposure of U937 cells to oxLDL did not trigger a conventional apoptosis response, but still resulted in phosphatidylserine externalization.

Keywords: Apoptosis; Cell death; U937; THP-1; Caspase; Oxidized low density lipoprotein

1. Introduction

The presence of a necrotic core is a key feature in advanced atherosclerotic plaques. This necrotic core region appears to be formed through the death of both macrophages and smooth muscle cells deep within the plaque [1–3]. The lysis of these cells results in the release of cellular components and may be the source of the cholesterol deposits found within advanced atherosclerotic plaques.

The oxidized low density lipoprotein (oxLDL) has been implicated as a key initiator in a number of plaque promoting processes. OxLDL has been shown to be taken up by macrophages in a rapid and uncontrolled manner leading to the formation of cholesterol filled foam cells, the major cellular component of fatty streaks. OxLDL is also cytotoxic, causing both necrosis and apoptosis in a variety of cell types including macrophages [4–6]. The change from fatty streak to complex plaque may be driven in part by the oxLDL induced death of macrophages within the plaque [1].

The exact mechanism of oxLDL cytotoxicity is difficult to define. OxLDL contains a number of known cytotoxic agents including oxysterol, reactive aldehydes such as 4-hydroxynonenal (HNE), and various protein derivatives all of which have been shown to cause macrophage cell death [7,8]. OxLDL also can cause intracellular oxidative stress by downregulating antioxidant enzymes or increasing the production of reactive oxygen species such as hydrogen peroxide [9].

In sufficient concentrations, oxLDL will cause macrophage cells to either undergo necrosis or apoptosis. Necrosis is characterized by cytoplasmic swelling, disruption of internal organelles, membrane lysis and release of cell debris into the extracellular space. In contrast, apoptosis is an active and highly regulated process resulting in the controlled shut down of the cell with the formation of small membrane bound vesicles that can be taken up by phagocytes. The features of apoptosis include morphological changes such as chromatin condensation and margination, DNA fragmentation and apoptotic body formation [5]. Typical biochemical changes comprise cytochrome *c* release from mitochondria, activation of cysteine-dependent caspases and labeling of the cell for clearance by phagocytes through exposure of phosphatidylserine on the surface of the cell [10]. In the absence of other cells to engulf the apoptotic cells, secondary necrosis may follow apoptosis [11].

The presence of caspase activation alone is often considered definitive evidence of apoptosis, since their cleavage of structural and regulatory proteins is responsible for the irreversible dismantling of the cell. Caspase activation has been found, or implied through use of enzyme inhibitors, in THP-1 cells incubated with oxLDL [10] and specific caspase assays in U937 cells incubated with hypochlorite-oxidized LDL [12]. In contrast, there is a growing awareness that apoptosis can proceed in the absence of caspase activation, and there can be differences in the quantification of apoptosis depending on the parameter being assessed [13]. This makes it often difficult to compare different studies when only a limited number of apoptosis markers are measured.

Here, we report on our investigation into the cytotoxicity of oxLDL in two different human monocytic cell lines, THP-1 and U937 cells, using a variety of apoptosis markers. Phosphatidylserine exposure occurred in both cell types, but caspase activation was only detected in the THP-1 cells. Absence of caspase activity in the U937 cells was associated with a dramatic decrease in intracellular thiols upon treatment with oxLDL.

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Abbreviations: LDL, low density lipoprotein; oxLDL, oxidized low density lipoprotein; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate

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2. Methods and materials

All chemicals used were of AR grade or better and obtained from the Sigma Chemical Company (USA) or BDH Chemicals New Zealand Limited unless otherwise indicated. Tissue culture media and plasticware were supplied by Gibco (USA) through Life Technologies (NZ). DTT was supplied by Boehringer–Mannheim. All solutions were prepared using ion-exchanged ultra filtered water prepared in a NANOpure ultrapure water system from Barnstead/Thermolyne (IA, USA).

2.1. Cell culture

THP-1 and U937 monocyte cultures were maintained in RPMI 1640 with 2 mM glutamine, 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere with 5% CO_2 at a density of not more than 1×10^6/ml. During experiments, cells were incubated for up to 48 h in RPMI 1640 alone at 5×10^5 cells/ml with or without native or oxLDL before washing in warm sterile phosphate-buffered saline (PBS) to remove the oxidant before analysis.

2.2. OxLDL preparation

EDTA-plasma was prepared from blood drawn by venipuncture from fasting normolipidemic volunteers. LDL was isolated by a single 22-h ultracentrifugation using a four step discontinuous gradient in a Beckman SW41 rotor. The LDL molar concentration was determined by enzymatic cholesterol determination using the ''Chol MPR 2'' kit supplied by Roche Chemicals (New Zealand) assuming an LDL molecular weight of 2.5 MDa and a cholesterol content of 31.6% [14]. Purified LDL was desalted by dialysis for 18 h against nitrogen-gassed chelex-treated PBS (pH 7.4) and oxidized by incubation with 50 μM $CuSO_4$ solution for 24 h. Native and oxLDL were concentrated with Urifil-10 concentrators (Millipore, MA, USA) and sterilized through a 0.22 μm membrane filter (Pall Gelman Laboratory) before addition to the cells.

2.3. Cell viability assays

The viability of the cells were measured using the MTT reduction and the trypan blue exclusion assays [15]. MTT reduction analysis was carried out using the method of Mosmann [16] but using 10% w/v sodium dodecyl sulfate (SDS, final concentration) to lyse the cells and solubilize the insoluble MTT-formazan salt.

2.4. Phosphatidylserine exposure and DNA fragmentation

Cells (10^5) were pelleted and suspended in binding buffer containing Annexin V-FITC and propidium iodide (PI), as per the manufacturer's instructions (Apoptest-FITC A700 kit, Nexins Research). Following 10 min in the dark, 10 000 cells were analyzed with a Vantage fluorescence-activated cell sorter from Becton–Dickinson (San Jose, CA). The cells were classified as viable, apoptotic or necrotic based on regions drawn on the dot plots. Cells with low Annexin V-FITC and PI fluorescence were classified as viable, cells binding Annexin V-FITC but excluding PI were classified as apoptotic and double labeled cells were classified as necrotic [17].

To stain the cellular DNA, cells (10^6) were pelleted and fixed in ice cold 70% ethanol. The cells were then washed in PBS and incubated at room temperature in 0.2 mM sodium phosphate containing 7 mM citric acid (pH 7.8). After pelleting and washing in PBS, the cells were suspended in 250 μl of PBS to which 10 μg RNAse A and 5 μg propidium iodide were added. Following 10 min in the dark 20 000 cells were analyzed using the fluorescence-activated cell sorter.

2.5. Caspase-3 activity

After treatment, samples of 10^5 cells were pelleted by centrifugation and stored at −80 °C before analysis. For caspase analysis 100 μl of buffer, containing 100 mM HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)), 10% sucrose, 0.1% Chaps (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), 10^{-4}% NP-40, 5 mM DTT and 50 μM DEVD-AMC (acetyl-asp-glu-val-asp-7-amido-4-methyl-coumarin) at pH 7.25 was added to each pellet [18]. The cleavage of the peptide substrate was monitored over 2 min by measuring the increase in fluorescence over time in a heat-jacketed F-4500 Hitachi fluorometer at λ_{ex} 370 nm (band width 5 nm) and λ_{em} 445 nm (band width 10 nm) at 37 °C. Fluorescence units were converted to pmol of free AMC using a standard curve generated with reagent AMC.

The conversion of pro-caspase-3 to its active form was measured by western blotting with a mouse monoclonal caspase-3 antibody (E-8) from Santa Cruz Biotechnology (CA, USA), in combination with a peroxidase-linked anti-mouse IgG and ECL-2 chemiluminescence from Amersham Bioscience NZ Ltd (Auckland, NZ).

2.6. Cellular reduced thiol concentration

After treatment cells were washed in cold PBS, then lysed by suspension and sonication in water. SDS (final concentration of 10%) and 5,5′-dithiobis (2-nitrobenzoic acid) [19] (DTNB, final concentration 30 μM) was added to the lysate and incubated at room temperature for 30 min before measuring the absorbance at 412 nm [20]. This analysis measures total cellular thiol content.

2.7. Statistical analysis

All values in the text and figures are expressed as means ± S.D. of triplicate treatments. Unless stated otherwise, results shown are from single experiments, representative of a minimum of three. The data were analyzed using the Statistica software package (Statsoft Inc., USA).

3. Results

U937 or THP-1 cells were incubated in serum free RPMI 1640 with increasing concentrations of oxLDL for 48 h (Fig. 1). Signs of toxicity were first evident in the MTT reduction assay and trypan blue exclusion assay at 0.5 mg/ml oxLDL, culminating in a 75% loss of viability at 3 mg/ml. In contrast, native (unoxidized) LDL at 1.5 mg/ml actually protected the U937 cells from the background toxicity attributable to culturing the cells in serum free media (Fig. 1).

Observation of morphological changes in the treated monocytes suggested that while death was the final endpoint in both cell lines, the processes leading to a loss in viability were different (Fig. 2). In particular, THP-1 cells showed signs of membrane blebbing and cell shrinkage 24 h after treatment (Fig. 2). In contrast, U937 cells had a more swollen appearance during the first 24 h after treatment, with the plasma membrane more rounded than that of untreated cells (Fig. 2F and G versus E). Cellular destruction was clearly visible at 48 h (Fig. 2H). These observations suggested that THP-1 cells were undergoing apoptosis, but a necrotic death was occurring in U937 cells.

THP-1 and U937 cells were incubated with oxLDL for 48 h and analyzed by flow cytometry using Annexin V-FITC binding to measure phosphatidylserine externalization. Co-labeling with propidium iodide allowed differentiation between apoptosis and necrosis, with control cells present in the lower left quadrant, apoptotic cells in the lower right quadrant and necrotic cells in the upper quadrants (Fig. 3A and B). Treatment of the THP-1 cells with 0.5 mg/ml oxLDL caused a decrease in viable cells with a rise in both phosphatidylserine externalized apoptotic cells and necrotic cells (Fig. 3C). Higher oxLDL concentrations caused the majority of the THP-1 cells to become necrotic. In contrast, the U937 cells showed little change in phosphatidylserine externalization and propidium iodide staining at 0.5 mg/ml oxLDL, but at 1.5 mg/ml oxLDL there was a large loss in control cells and an increase in both apoptotic and necrotic cells (Fig. 3D). At 3 mg/ml oxLDL all the U937 cells were necrotic. A cell cycle analysis was undertaken

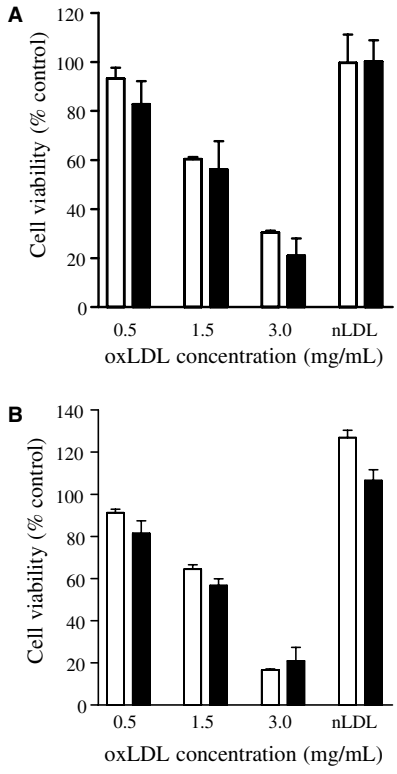

Fig. 1. OxLDL decreases cell viability of human monocyte cell lines. THP-1 cells (A) and U937 cells (B) (5×10^5cells/ml) were incubated with varying concentrations of OxLDL in serum free RPMI 1640. Cell viability was determined after 48 h by MTT reduction (open bars) and trypan blue exclusion analysis (solid bars). A non-oxidized LDL (nLDL) at 1.5 mg/ml was included as an additional control. The results are expressed as a mean percentage of triplicates ± S.D. of control cells incubated in LDL free media.

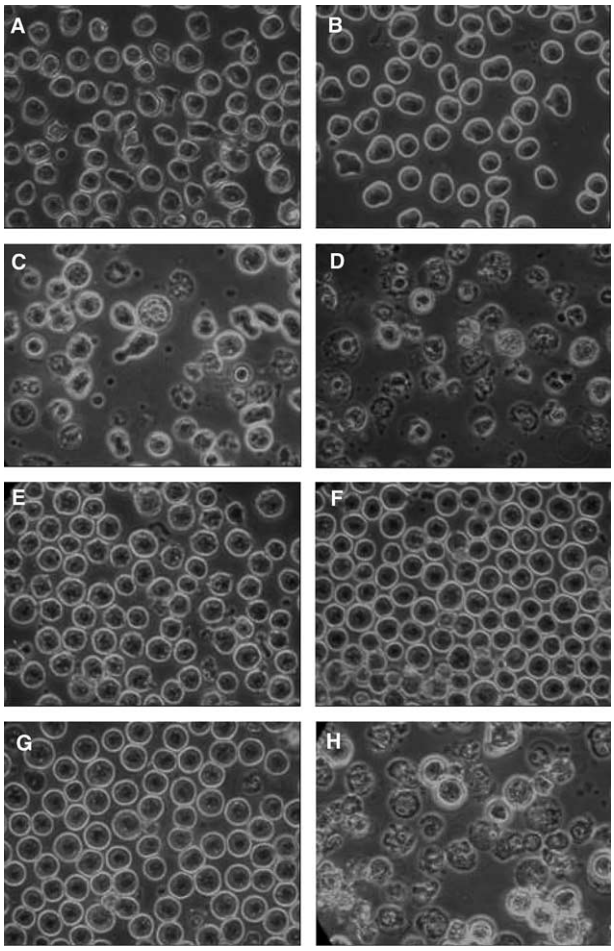

Fig. 2. Morphological changes in monocytes treated with oxLDL. THP-1 cells (A–D) and U937 cells (E–H) were incubated with 1.5 mg/ml OxLDL for 0 (A,E), 6 (B,F), 24 (C,G) and 48 (D,H) h before being photographed with a reversed-phase light microscope.

to determine if oxLDL caused DNA fragmentation in the cells (Fig. 3E–H). In U937 cells incubated with 1.5 mg/ml oxLDL there was a loss of cells in the G_0/G_1 phase, with an increase of cells in the G_2/M (Fig. 3G and H). Very few cells were present in the sub G_0/G_1 fraction, dispite 25% of the cells being phosphatidylserine-positive after 48 h (Fig. 3D). We therefore conclude that oxLDL is not triggering DNA fragmentation in the U937 cells.

Measurement of caspase-3 like DEVDase activity showed that oxLDL caused a significant and concentration dependent increase in caspase activation in THP-1 cells (Fig. 4A). The rate of activation was dependent on the oxLDL concentration with 0.2 mg/ml oxLDL causing activation between 24 and 48 h while a significant rise in activity was observed with 3 mg/ml oxLDL after only 6 h. With 0.5 and 1.5 mg/ml oxLDL there was no significant rise in caspase-3 activity until after 12 h of incubation. At the lower concentrations of oxLDL (0.2–0.5 mg/ml), caspase activation was continuing to increase over the 48 h, consistent with optimal PS exposure observed with these concentrations of oxLDL. The two highest doses of oxLDL (1.5–3 mg/ml) caused the caspase activity to peak and then fall. This profile, along with the flow cytometry data (Fig. 3C), suggests the higher concentrations of oxLDL cause a shift from apoptosis to secondary necrosis. In dramatic contrast to the THP-1 cells, there was no major increase in caspase activity in the U937 cells treated with oxLDL (Fig. 4B). Instead, at concentrations of 0.5 mg/ml and greater there was a small but significant decrease in background caspase activity, first detectable 12 h after treatment. Consistent with the activity assays, Western blotting showed conversion of pro-caspase-3 to its active form only occurred in the THP-1 cells (Fig. 4C).

Decreased caspase activity has been observed in cells placed under excess oxidative stress, consistent with the observation that the active site cysteine of the caspases has to be reduced to be active [21,22]. To explore this possibility, we measured total thiol levels in the cells following treatment with oxLDL. This analysis measured the combined cellular glutathione and protein thiols content. There was a concentration-dependent loss in thiols in THP-1 cells (Fig. 4D), but this was consistent with the loss in cell viability (Fig. 1). In contrast, there was a more marked loss of reduced thiols in the U937 cells. At 1.5 mg/ml oxLDL there was an almost complete loss of reduced thiols (Fig. 4D), yet a large number of U937 cells retained their viability (Fig. 1). A time course showed that the U937 cells lost their intracellular thiols between 6 and 12 h after the addition of the oxLDL (Fig. 4E). This loss was closely paralleled by a decrease in basal caspase activity suggesting that oxLDL induced stress was interfering with caspase activation in the U937 cells.

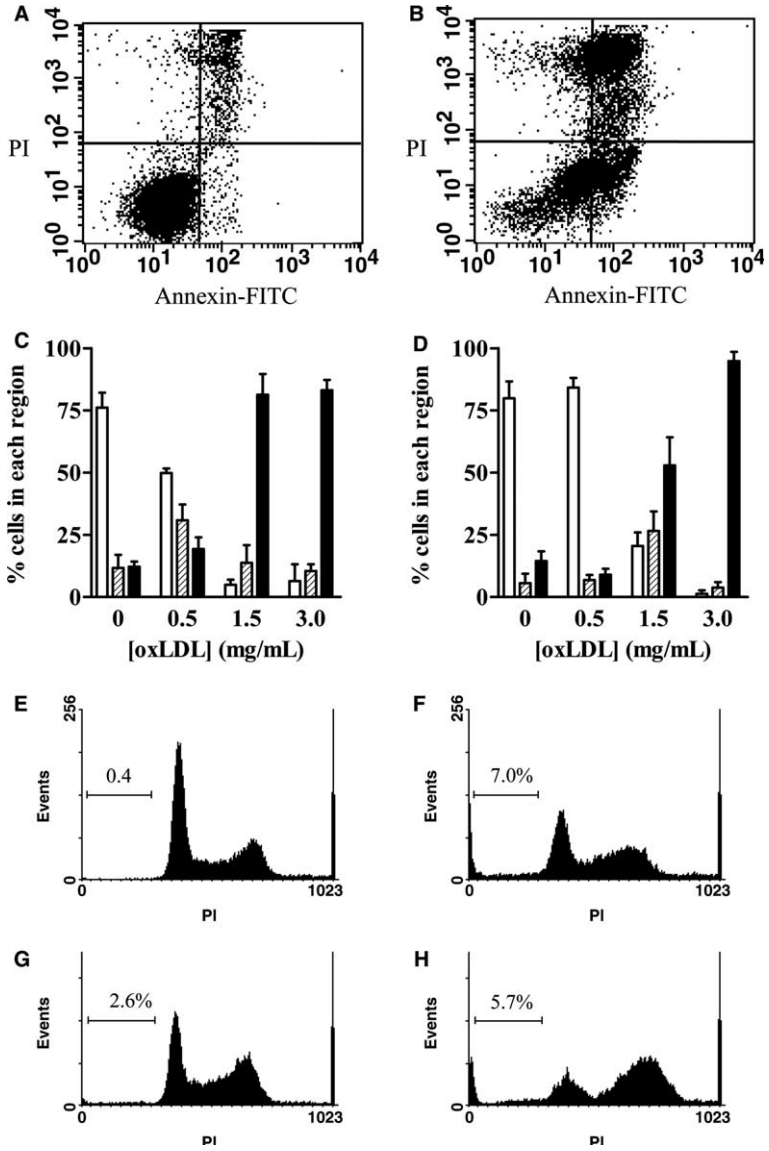

Fig. 3. Flow cytometery analysis of oxLDL-treated cells. Cells were incubated for 48 h at 5×10^5 cells/ml in RPMI 1640 with or without oxLDL, before staining and analysis on a FACS Vantage flow cytometer. To assess phosphatidylserine exposure, the cells were co-stained with annexin V-FITC and propidium iodide. A representative experiment showing 5000 U937 cells treated with (A) 0 and (B) 1.5 mg/ml oxLDL is presented. OxLDL was added to (C) THP-1 and (D) U937 cells at a range of concentrations. The open bars represent control cells, hatched bars the apoptotic cells and solid bars the necrotic cells. Values are the means ± S.D. of three independent experiments. To measure cellular DNA degradation THP-1 cells (E,F) or U937 (G,H) cells with (F,H) or without (E,G) 1.5 mg/ml oxLDL were incubated for 48 h in RPMI 1640. The cells were fixed and their DNA was stained with propidium iodide before analysis by flow cytometry.

4. Discussion

In this study, we have shown that oxLDL triggers apoptosis in monocytic cell lines, but the nature of the apoptotic program varies dramatically depending on the cell type. In the THP-1 cells, apoptosis proceeds via a conventional pathway that includes caspase activation, phosphatidylserine exposure, and finally a loss in cell viability with a corresponding decline in intracellular reduced thiols. In contrast, U937 cells show an early decline in reduced thiols and phosphatidylserine exposure occurs in the absence of any discernable caspase activation.

U937 cells have a functional caspase network that is activated by a range of apoptotic stimuli [23,24]. Consistent with our results, Lizard et al. [25] observed only minor cleavage of pro-caspase-3 after incubation of U937 cells with 7-ketocholesterol, a component of oxLDL. However, Vicca et al. [12] were able to measure significant caspase activity in U937 cells treated with hypochlorous acid-oxidized LDL, suggesting that this may act differently from the Cu-oxidized LDL used in this study.

The response of the U937 cells is similar to what has been observed previously with stimulated neutrophils. Products of the neutrophil oxidative burst trigger phosphatidylserine exposure, but do so in the absence of caspase activation [26]. Caspases have a redox-sensitive cysteine at their catalytic site, and oxidation of this residue may prevent auto-proteolytic activation of the caspase cascade. Dithiothreitol is included with

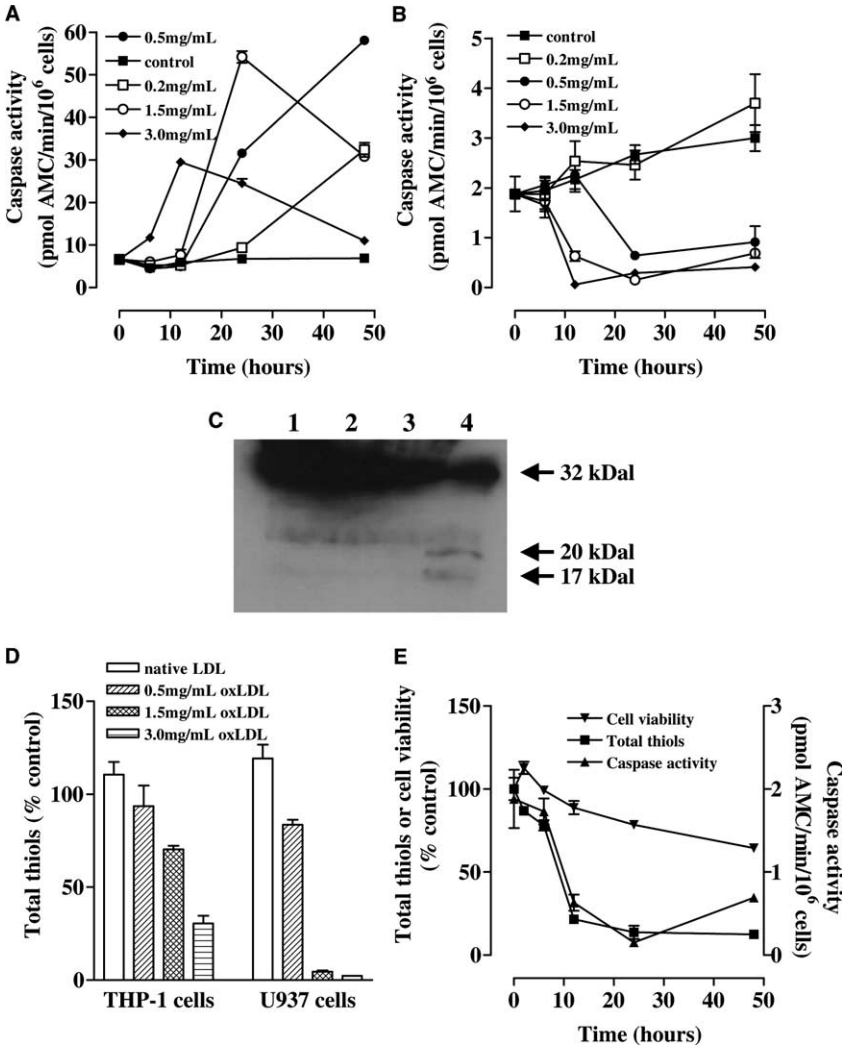

Fig. 4. Caspase activation and thiol loss in monocytes treated with oxLDL. THP-1 (A) and U937 cells (B) were incubated in RPMI 1640 with varying concentrations of oxLDL for up to 48 h. The cells were washed, caspase buffer added and the cleavage of the substrate monitored as increase in fluorescence over time at 37 °C. Fluorescence units were converted to pmol of free AMC using a standard curve generated with reagent AMC. (C) A Western blot for caspase-3 is present showing: Lane 1 = untreated U937 cells; lane 2 = U937 cells with 1.5 mg/ml oxLDL for 24 h; lane 3 = untreated THP-1 cells; lane 4 = THP-1 cells with 1.5 mg/ml oxLDL for 24 h. Arrows indicate the active 17 kDal and 20 kDal forms of caspase-3. The 32 kDal band for pro-caspase-3 is overexposed to enable visualization of the active forms. (D) THP-1 and U937 cells were incubated at 5×10^5cells/ml in RPMI 1640 for 48 h with or without native or oxidized LDL and the levels of reduced total thiols analyzed using the DTNB thiol assay. (E) Time course of U937 cell viability (MTT reduction), caspase activity and total cellular thiol loss over 48 h. Values are expressed as the means ± S.D. of triplicates.

purified caspases to maintain catalytic competency [27], and the enzymes are also sensitive to inactivation by a range of reactive oxygen and nitrogen species [22,28]. Alternatively, other redox-sensitive proteins critical for the initiation of apoptosis may be prevented from functioning.

Components of the oxLDL could selectively oxidize cellular thiol proteins, or promote global oxidation by impacting on the glutathione- and thioredoxin-dependent reduction pathways. In U937 cells the loss of reduced thiols and the inactivation of caspases by oxLDL occurred in the same pattern over time, indicative of global intracellular oxidation (Fig. 4). The loss of all background caspase activity in the U937 cells treated with oxLDL emphasizes the intensity of the oxidative stress present in these cells.

The difference in response between U937 and THP-1 cells to oxLDL is intriguing and may relate to varying mechanisms of uptake of the oxLDL. U937 cells express fourfold higher levels of CD36 scavenger receptor than do THP-1 cells [29]. This may result in an initial burst of oxLDL uptake and a more intensive oxidative stress. The two cell lines also have other noted differences in their response to oxidative stress. U937 cells do not express the transcriptional regulator peroxisome proliferator-activated receptor-γ in response to oxLDL [30], as THP-1 cells do. This response is believed to be mediated via the oxidized lipids in copper-oxidized LDL. U937 cells also have a lower level of glutathione expression but higher MnSOD than THP-1 cells [31]. How this will affect the potential cellular stress induced by the oxLDL is difficult to determine from the current literature.

In conclusion, oxLDL is able to trigger different forms of apoptosis, even between two human monocytic cell types. The difference in response is likely to depend upon the

handling of the oxLDL, and the redox environment of the target cell. We suggest that the exact pathways of apoptotic cell death initiated by oxLDL may vary in the atherosclerotic lesion depending on the cell type and the local environment.

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