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Fragmentation of DNA in P388D$_1$ macrophages exposed to oxidised low-density lipoprotein

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Exposing a macrophage-like murine cell line to copper-oxidised low-density lipoprotein led to DNA fragmentation which was inhibited by the putative Ca^{2+}/Mg^{2+} endonuclease inhibitor, zinc sulphate. DNA fragmentation preceded loss of membrane impermeability. These results suggest that apoptosis may be a mechanism of macrophage foam cell death in atherosclerotic lesions in the arterial wall.

Oxidised LDL; Macrophage; DNA fragmentation; Apoptosis; Atherosclerosis

1. INTRODUCTION

The role of the macrophage in atherosclerosis is of increasing interest since the observations of their uptake in vitro of modified low-density lipoprotein (LDL) [1], including oxidised LDL (oxLDL) [2], to form lipid-laden foam cells resembling those in atherosclerotic lesions. In advanced lesions these foam cells appear to die, giving rise to the characteristic necrotic core [3], and studies of macrophages in culture have suggested that their death may be due to their ability to oxidise LDL [4].

OxLDL is toxic in vitro to all the cell types found in atherosclerotic lesions, including endothelial and smooth muscle cells [5,6] and macrophages [4].

Apoptosis is an active process of cell death distinct from necrosis which was first described in developing and stable tissues as 'programmed cell death'. Ultrastructural features of apoptotic cells include vacuolation of the endoplasmic reticulum, cellular condensation and formation of characteristic blebs of the cell surface [7–9], all of which are observed in macrophages exposed to oxLDL [10]. Chromatin cleavage, characterised by double-stranded DNA fragmentation [9], is often used as an indicator of apoptosis. DNA fragmentation is thought to be caused by the activation of Ca^{2+}/Mg^{2+}-dependent endonucleases [11,12]. It is not always physiological [13] since many injurious agents have been shown to be able to induce apoptosis in various cell types [14–16].

We have exposed P388D$_1$ cells to copper-oxidised LDL, to investigate the effects of oxLDL on (i) cell viability, by measurement of cell membrane permeability to adenine nucleotides, and (ii) chromatin structure in the presence and absence of zinc sulphate (Zn^{2+}) as an inhibitor of endonuclease activity.

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2. MATERIALS AND METHODS

Radiochemicals were obtained from Amersham International (Aylesbury, UK). All biochemicals were obtained from Sigma Chemical Co. (Poole, UK) unless stated otherwise and were of the highest purity available. DNA molecular weight marker III, Lambda DNA *Eco*RI and *Hin*dIII was obtained from Boehringer-Mannheim Biochemica (Lewes, UK).

LDL was isolated from normal human serum as described previously [3]. Oxidation was carried out with copper sulphate (5 μM) at 37°C for 24 h. Ethylenediaminetetraacetic acid (EDTA) and copper sulphate were removed from the LDL and oxLDL, respectively, using a G-10 Sephadex PD10 column. Peroxidation of the LDL was confirmed using the Xylenol orange assay [17], and protein content measured by the method of Lowry et al. [18].

The macrophage-like P388D$_1$ cell line was cultured in 24-well plates (Becton Dickinson and Co., CA, USA) for adenine nucleotide leakage and DNA fragmentation assays at 3×10^5 cells per well overnight before the start of the experiment and in 60×15 mm tissue culture dishes (Becton Dickinson and Co.) for DNA fragmentation experiments at 3×10^6 cells per dish. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% lipoprotein-deficient fetal calf serum, [19] penicillin (100 IU/ml) and streptomycin (100 μg/ml).

Increases in membrane permeability were determined using tritiated adenine as described previously [20,21]. Briefly, the cells were pre-loaded with 1 μCi/ml tritiated adenine for 1 h at 37°C, then washed 3 times in PBS (pH 7.4). Fresh medium was added. Cells were then exposed to oxLDL (100 μg/ml). No addition controls (NA) were also prepared in which the loaded cells were incubated in culture medium alone. Increased permeability was calculated from the percentage release of tritiated adenine nucleotides from the cells at the end of each experiment.

DNA fragmentation was quantified by tritiated thymidine release as described previously [22]. Cells were pre-loaded with 1 μCi/ml tritiated thymidine for 24 h at 37°C, washed with PBS (pH 7.4) and left on ice for 1 h. Fresh medium was then added to the cells. OxLDL was added at 100 μg/ml. At the end of the experiment the medium was collected and the cells were lysed in 1 ml of 30 mM sodium acetate, pH 6.6. The lysates were centrifuged at $15{,}000 \times g$ for 30 min to

separate intact chromatin (pellet) from fragmented DNA (supernatant). Radioactivity in 200 μl of incubation medium, supernatant and pellet was determined. Specific DNA fragmentation was calculated from:

$$\% \text{ specific DNA fragments} = 100 \times \text{cpm}_{\text{frags}}/\text{cpm}_{\text{total}}$$

where $\text{cpm}_{\text{frags}}$ = cpm in incubation medium + cpm in supernatant, and $\text{cpm}_{\text{total}} = \text{cpm}_{\text{frags}}$ + cpm in the pellet.

DNA agarose gels were used to visualise DNA fragmentation. The cells were harvested and centrifuged for 10 min at 1,000 × g. The cell pellet was lysed in 0.2 ml lysis buffer containing 10 mM Tris-HCl, 1 mM EDTA and 1% sodium dodecyl sulphate (SDS; v/v) at pH 7.5. The samples were incubated in the lysis buffer for 15 min at 37°C. Proteinase K was then added at 100 μg/ml and incubated for 2 h at 37°C. DNA was extracted with phenol, phenol-chloroform and then chloroform, with centrifugation steps between extractions for 10 min at 15,000 × g. DNA was precipitated by first adding sodium acetate to a final concentration of 0.3 M followed by adding twice the resulting volume of isopropanol. The samples were stored at −70°C for at least 1 h. Samples were centrifuged for 20 min at 15,000 × g, the pellets washed in 70% (v/v) ethanol and allowed to dry. Pellets were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) to which loading buffer (50% glycerol (v/v), 5 × TAE, 0.1% Bromophenol blue (w/v)) was added.

Electrophoresis was carried out on 1.8% agarose gels (w/v) made in 1 × TAE buffer (40 mM Tris acetate to pH 8.5 and 2 mM EDTA). DNA was stained with ethidium bromide (5 μg/ml) and then treated overnight with ribonuclease (RNase: 1 μg/ml). The RNase was incubated at 65°C for 10 min to inactivate any deoxyribonuclease (DNase) activity. The DNA was visualised on a UV transilluminator.

In some experiments zinc sulphate (1 mM) was added to the cell cultures at the same time as the addition of the oxLDL.

All experiments were carried out for a minimum of three times in duplicate or triplicate. Results were evaluated using the paired Student's *t*-test and the values are expressed as mean ± S.D.

3. RESULTS AND DISCUSSION

The initiation of DNA fragmentation was found to be an early event in $P388D_1$ exposed to oxLDL, occurring prior to changes in the release of adenine nucleotides, i.e. before changes in membrane integrity. This contrasts with earlier findings that oxLDL induced permeability changes in lymphoblastoid cells which occurred in parallel to DNA fragmentation [23].

Exposure of $P388D_1$ cells to oxLDL did not cause detectably increased membrane permeability up to, and including, 24 h. However, adenine nucleotide release was significantly above the values exhibited by control incubations at 36 and 48 h ($P < 0.001$; Fig. 1). This increased membrane permeability was not ameliorated in the presence of Zn^{2+} (data not shown).

OxLDL produced significantly greater DNA fragmentation than in control cells at 6 h ($P < 0.01$) which increased markedly after 36 h (Fig. 2). The extent of fragmentation was significantly reduced in the presence of Zn^{2+} at 6 h ($P < 0.01$), 36 h ($P < 0.5$) and 48 h ($P < 0.001$).

Agarose gels of DNA extracted from $P388D_1$ cells incubated with oxLDL revealed oligonucleosomal fragmentation with the characteristic ladder pattern associated with apoptosis. This oligonucleosomal chromatin

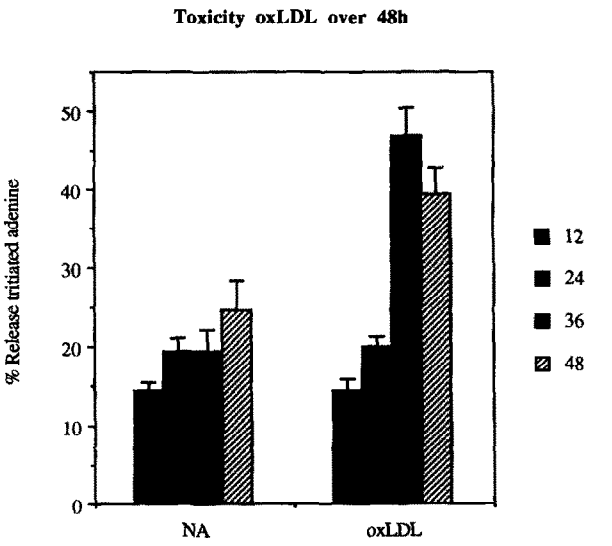

Fig. 1. Tritiated adenine nucleotide leakage from $P388D_1$ cells incubated with 100 μg/ml oxLDL over 48 h compared to no addition (NA) control cells.

cleavage was absent in control cells and in cells exposed to oxLDL in the presence of Zn^{2+} (Fig. 3). This confirmed that the DNA fragmentation seen was double-stranded and that the cleavage was dependent on the activity of the Ca^{2+}/Mg^{2+}-dependent-endonuclease, similar to that seen in cells undergoing apoptosis.

Our findings with respect to the Ca^{2+}/Mg^{2+}-dependent endonuclease inhibitor, Zn^{2+}, do not conflict with the hypothesis that apoptosis is a mechanism of oxLDL-induced cell killing. Ueda and Shah [24] showed recently that endonuclease inhibitors, including Zn^{2+}

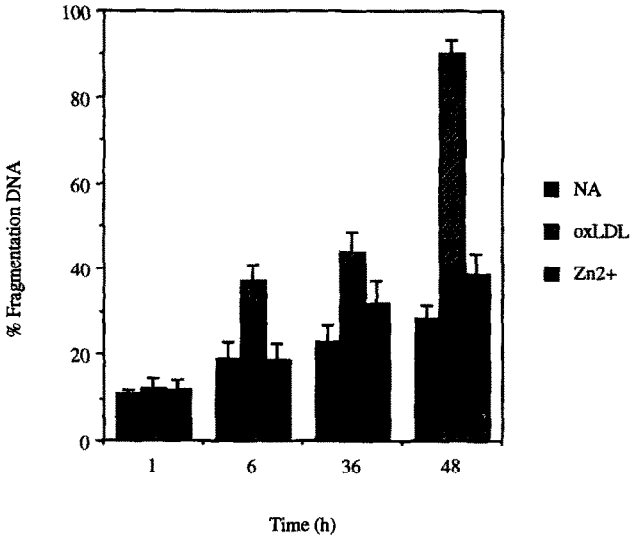

Fig. 2. DNA fragmentation measured by tritiated thymidine radioactivity in medium and centrifuge supernatants (see section 2). $P388D_1$ was incubated for 1–48 h with 100 μg/ml oxLDL with and without addition of 1 mM Zn^{2+} and compared to no addition (NA) control cells.

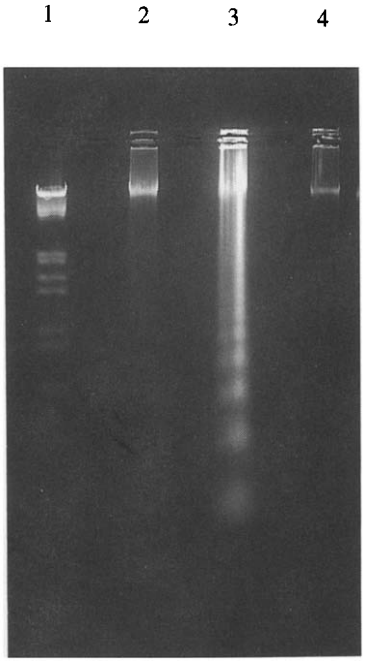

Fig. 3. Agarose gel electrophoresis of DNA extracted from P388D$_1$ after 24 h incubation with 100 μg/ml oxLDL with and without 1 mM Zn^{2+}. Lane 1, molecular weight markers (λ-phage DNA cut with *Hin*dIII and *Eco*RI); lane 2, no addition control cells; lane 3, oxLDL; lane 4, oxLDL and zinc sulphate.

and aurintricarboxylic acid, protected a renal tubular epithelial cell line against chromatin cleavage by hydrogen peroxide. However, Zn^{2+} did not protect thymocytes against dexamethasone-induced apoptosis [25], although it reduced internucleosomal cleavage. High-molecular weight fragments, induced by dexamethasone, were found in these experiments, the presence of which were unaffected by the addition of Zn^{2+}. It was concluded that Zn^{2+} does not inhibit dexamethasone-induced apoptosis in thymocytes and that key enzymes other than the Ca^{2+}/Mg^{2+}-dependent endonuclease, are involved in induction of the early stages of apoptosis. Our findings are consistent with this hypothesis. In the P388D$_1$ exposed to oxLDL, Zn^{2+} inhibited oligonucleosomal chromatin cleavage but did not reduce toxicity.

It is clear from these results that oxLDL can cause chromatin changes characteristic of apoptosis in a macrophage-like cell line. The finding that oxLDL can induce apoptosis may be important in understanding the mechanism of macrophage death in the advancing atherosclerotic plaque.

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