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Ascorbate does not protect macrophages against apoptosis induced by oxidised low density lipoprotein

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Short title: Oxidised LDL, ascorbate and macrophage apoptosis

Abstract

Apoptosis of macrophages and smooth muscle cells is observed in atherosclerotic lesions and may play an important role in the disease progression. Oxidised low density lipoprotein (LDL) is cytotoxic and induces apoptosis in a variety of cell types. We reported previously that ascorbate protects arterial smooth muscle cells from apoptosis induced by oxidised LDL containing the peak levels of lipid hydroperoxides. We now demonstrate that macrophages undergo apoptosis when treated with this species of oxidised LDL, as detected by increased annexin V binding and DNA fragmentation. Ascorbate treatment of macrophages did not protect against the cytotoxicity of oxidised LDL, and modestly increased the levels of annexin V binding and DNA fragmentation. Oxidised LDL treatment also increased the expression of the antioxidant stress protein heme oxygenase-1 in macrophages; however, this increase was markedly attenuated by ascorbate pretreatment. Although apoptosis induced by oxidised LDL was modestly promoted by ascorbate, ascorbate apparently decreased the levels of oxidative stress in macrophages, suggesting that this pro-apoptotic effect was not mediated by a pro-oxidant mechanism, but may instead have been due to intracellular protection of the apoptotic machinery by ascorbate.

Key words: apoptosis, ascorbate, atherosclerosis, macrophage, oxidised LDL

1. Introduction

Atherosclerosis, leading to coronary heart disease and strokes, is the leading cause of death in the western world. Atherosclerotic plaque formation is characterised by accumulation of lipid within the vessel wall, inflammation, local changes in structure and cell death [1]. Apoptosis within atherosclerotic lesions has been well documented [2, 3], and macrophage apoptosis may be of importance in lesion progression [4]. Macrophage apoptosis may be beneficial by preventing foam cell formation and release of inflammatory cytokines and matrix metalloproteinases into the vessel wall [5]. On the other hand, phosphatidylserine externalised by apoptotic cells is thrombogenic [6] and if removal of apoptotic cells from lesions becomes compromised, secondary necrosis may ensue, leading to the release of growth factors, cytokines, proteases and intracellular lipid into the extracellular space. These factors may serve to propagate inflammation and extracellular lipid deposition within the atherosclerotic lesion, thus promoting disease progression [5].

Oxidised LDL is implicated in the pathogenesis of atherosclerosis [1, 7] and is associated with apoptosis of vascular cells in human atherosclerotic plaques [8]. Indeed, oxidised LDL is known to be cytotoxic to a variety of cell types including endothelial cells, smooth muscle cells, macrophages and lymphocytes [9-13]. We have shown using LDL oxidised to defined extents that LDL containing the maximum level of lipid hydroperoxides (LOOH), termed moderately-oxidised LDL, can induce apoptosis in human umbilical artery smooth muscle cells, whereas native LDL does not [14]. However, we now demonstrate that although moderately-oxidised LDL induced apoptosis in macrophages, the LDL species most toxic towards this cell type is a highly-oxidised LDL preparation, rich in 7-ketocholesterol.

Ascorbate is a highly effective water-soluble antioxidant, capable of scavenging a

variety of free radical species and oxidants. Ascorbate supplementation of cholesterol-fed animals has been shown to be anti-atherogenic [15-17], although other studies have reported no significant biological effect [18, 19]. Some epidemiological studies in man [20, 21], but not all [22], have shown that individuals who consume high concentrations of ascorbate through diet or supplements tend to have lower risks of cardiovascular disease, however, large clinical trials have found no benefits of antioxidant supplementation on cardiovascular disease risk [23]. We and others have shown that antioxidants [24, 25], including ascorbate [14], afford protection to cells from the pro-apoptotic effects of oxidised LDL. This phenomenon has also been observed in macrophages [26, 27]. We report in this publication, however, that ascorbate treatment of macrophages conferred no protection against apoptosis induced by moderately-oxidised LDL and modestly, but significantly, augmented the induction of phosphatidylserine externalisation and DNA fragmentation in these cells.

Heme oxygenase-1 is a microsomal stress-induced protein that catalyses the rate-limiting step in the degradation of heme into equimolar quantities of biliverdin, carbon monoxide and iron [28]. Prominent expression of heme oxygenase-1 is observed in macrophage-derived foam cells present in atherosclerotic lesions [29], and heme oxygenase-1 is induced in smooth muscle cells and mouse peritoneal macrophages after treatment with oxidised LDL [29-31]. In the study reported here, we have used heme oxygenase-1 protein expression as an indicator of the levels of oxidative stress in macrophages treated with moderately-oxidised LDL.

2. Materials and methods

2.1. Isolation of LDL

LDL (density 1.019-1.063g/ml) was isolated from the pooled plasma of healthy volunteers by sequential density ultracentrifugation in the presence of EDTA, as described previously [32].

2.2. Oxidation of LDL (Method 1; 37°C)

Native LDL was diluted to 150µg protein/ml with sterile PBS (137mM NaCl, 2.68mM KCl, 1.47mM KH₂PO₄ and 8.1mM Na₂HPO₄; Gibco, Paisley, UK) and oxidised at 37°C by addition of CuSO₄ (5µM above EDTA concentration carried over from the LDL preparation, typically around 6µM in total). The extent of oxidation was monitored by following the formation of conjugated dienes, which absorb at 234nm [33]. Every 15 minutes an aliquot of LDL was removed and the absorbance at 234nm was measured before returning the aliquot to the sample. When the absorbance had increased by 0.2 above the starting value, one third of the LDL was removed, oxidation was terminated by addition of EDTA (1mM) and the sample was stored at 4°C. This LDL was defined as mildly-oxidised LDL. The remaining LDL was allowed to oxidise until the absorbance peaked ($A_{234}=2.0-2.6$, after approximately 3-4h). Half of this LDL was removed and again oxidation was terminated by addition of EDTA and the sample stored at 4°C. This LDL was designated moderately-oxidised LDL and contained the peak level of LOOH. The remaining third of the LDL was oxidised for 24 hours before EDTA was added, and was designated highly-oxidised LDL. The three oxidised LDL species were concentrated by ultracentrifugation. To do this, the density was adjusted to 1.1g/ml for mildly- and moderately-oxidised LDL, and 1.15g/ml for highly-oxidised LDL by addition of solid potassium bromide. The mass of potassium bromide required was calculated using the following equation [34]:

$$\text{Mass of KBr} = \text{volume of LDL sample (ml)} \times \frac{(\text{required density} - 1.006)}{1 - (\text{partial specific volume of KBr at final density} \times \text{required density})}$$

Transition metal ions were removed by the addition of washed Chelex-100. LDL was concentrated by ultracentrifugation at 115,000 g for 18 hours at 4°C. After collection by needle and syringe, LDL was dialysed in 8mm diameter dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA, USA) for 18 hours against four changes of 1.25L phosphate buffer (140mM NaCl, 8.1mM Na₂HPO₄, 1.9mM NaH₂PO₄, 100µM EDTA, pH 7.4) to remove residual potassium bromide and copper ions and to restore the concentration of EDTA to 100µM. The LDL species were filter-sterilised (0.2µm pore size) and stored at 4°C. A modified Lowry assay [35] was used to determine protein concentration and LOOH content was determined using the tri-iodide method of El-Saadani et al. [36].

2.3. Oxidation of LDL (Method 2; 4°C)

Native LDL was dialysed in 8mm diameter dialysis tubing against 3 changes of phosphate buffer (140mM NaCl, 8.1mM Na₂HPO₄, 1.9mM NaH₂PO₄, pH 7.4) for 4.5 hours at 4°C to remove EDTA from the LDL. This LDL was then dialysed overnight at 4°C against 2 changes of Chelex-treated MOPS buffer (10mM MOPS, 150mM NaCl, pH 7.4, 1g washed Chelex per litre). The LDL was then diluted to 2mg protein/ml with MOPS buffer containing CuSO₄ (final concentration 10µM) and transferred to fresh dialysis tubing (8mm diameter). It was then dialysed against stirred Chelex-treated MOPS containing 10µM CuSO₄ for 18 hours at 4°C to generate an oxidised LDL rich in LOOH. To terminate oxidation, EDTA (100µM) was added directly to the dialysis tubing and the LDL was dialysed against phosphate buffer containing EDTA (100µM) at 4°C for 24

hours. The oxidised LDL was filter sterilised (0.2µm pore size) and stored at 4°C, and the protein concentration and LOOH level were determined, as before [35, 36].

2.4. Cell culture

The murine macrophage cell line J774 was cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 20% (v/v) FBS, Glutamax (2mM), penicillin (50IU), streptomycin (50µg/ml) and amphotericin B (0.95µg/ml) at 37°C in a humidified 95% air/5% carbon dioxide atmosphere. In experiments examining the effects of ascorbate, cells were treated with ascorbate (100µM; Sigma-Aldrich Inc., Saint Louis, MO, USA) freshly prepared in PBS (Gibco). During pretreatment, cells were incubated for 24 hours with ascorbate in serum-containing medium, washed with PBS and reincubated with complete medium containing native or oxidised LDL in the absence of ascorbate. During cotreatment, cells were concurrently incubated with ascorbate and native or oxidised LDL. During pre- and cotreatment, cells were incubated for 24 hours with ascorbate in complete medium, this medium was removed, cells were washed with PBS and were then incubated with fresh medium containing freshly prepared ascorbate (100µM) and either native or oxidised LDL.

Mouse peritoneal macrophages were isolated from mice (129sv back crossed to C57BL/6) by peritoneal lavage, as described previously [37], and were cultured in DMEM supplemented with 10% (v/v) FBS, gentamicin (50µg/ml), penicillin (500IU/ml) and streptomycin (500µg/ml) at 37°C in a humidified 95% air/5% carbon dioxide atmosphere.

Human blood-derived monocytes were isolated using Nycoprep 1.068 solution (Robbins Scientific, Solihull, UK). Briefly, venous blood (100ml) was collected in the presence of EDTA (3mM) and transferred in 10ml aliquots to tubes containing 1ml of 6%

(w/v) dextran solution. The tubes were inverted several times and left standing at a 45° angle for 1 hour at room temperature. The leukocyte rich plasma layer that formed at the top of the tubes was collected and layered over the Nycoprep solution and centrifuged at 650 g for 15 minutes. The monocyte rich layer from each tube was collected, pooled and diluted with an equal volume of sterile PBS. These cells were centrifuged at 1500 g for 7 minutes and the pellet resuspended in 10ml PBS. The monocytes were again centrifuged at 1500 g for 5 minutes and the pellet resuspended in culture medium (RPMI (Gibco) containing 10% FBS, 2mM glutamine, and supplemented with penicillin (20IU/ml) and streptomycin (20µg/ml). The cells were cultured at 37°C in a humidified 95% air/5% carbon dioxide atmosphere, and after 4 hours the cells were washed 3 times to remove any contaminating lymphocytes (non-adherent).

2.5. Detection of apoptosis

2.5.1 DNA fragmentation

Cellular DNA fragmentation was measured using a commercially available cellular DNA fragmentation kit (Roche, Lewes, UK), according to the manufacturer's instructions. Briefly, proliferating cells on 96 well plates were labelled with 5-bromo-2-deoxyuridine (BrdU; 10µM) for 24 hours, washed with PBS, treated with or without ascorbate for 24h, and treated with oxidised LDL up to 48 hours. The growth medium was then removed and the cells incubated for 30 minutes at room temperature with kit lysis buffer (BSA, EDTA and Tween 20). Liberated BrdU-labelled DNA fragments were quantified by ELISA. The cell lysates containing the labelled DNA fragments were allowed to bind to an anti-DNA antibody adsorbed to the wells of a microtiter plate. After washing, the DNA fragments were denatured and fixed by microwave irradiation and detected by an anti-BrdU antibody-horseradish peroxidase conjugate. The amount of peroxidase bound to the immune complex was quantified by the addition of tetramethylbenzidine substrate and DNA

fragmentation was expressed as absorbance at 450nm.

2.5.2. Flow cytometry

An early marker of apoptotic cell death is the externalisation of phosphatidylserine to the cell surface [38]. This change was detected according to the manufacturer's instructions, using a commercially available FITC-annexin V conjugate (Clontech Laboratories Inc., Palo Alto, CA, USA), which binds to phosphatidylserine. Propidium iodide, a marker of membrane permeability, was used to distinguish apoptosis from necrosis, and flow cytometry was used to quantify the extent of apoptosis and necrosis. Briefly, proliferating cells were incubated with native or oxidised LDL for up to 48 hours. Cells were harvested in serum-containing growth medium and centrifuged. Pellets were resuspended in kit binding buffer and centrifuged again. These pellets were resuspended in 200µl of binding buffer containing annexin V (0.5µg/ml) and propidium iodide (2.5µg/ml) and were incubated for 15 minutes in the dark. Data acquisition and analysis were performed with a Becton Dickinson FACS-can flow cytometer using CellQuest software (Becton Dickinson, Oxford, UK).

2.5.3. MTT assay

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is reduced to formazan by mitochondrial dehydrogenase in healthy cells. The activity of this enzyme decreases as cells die. Proliferating cells cultured on 96 well plates were treated with native or oxidised LDL for up to 48h. The culture medium was then replaced with a 0.05% (w/v) MTT solution (Sigma-Aldrich) in fresh medium. Cells were incubated for 1 hour at 37°C. DMSO (100µl/well) was added to the microtiter plate to solubilise the formazan crystals formed, the plate was incubated on a shaker and absorbance was measured using a plate reader. Mitochondrial dehydrogenase activity was proportional to levels of formazan

generated and was expressed as absorbance at 540nm.

2.6. Western blotting

Proliferating cells were treated with native or oxidised LDL for up to 48 hours. Cells were washed with ice-cold PBS and lysates prepared with lysis buffer (1ml of buffer contains stock buffer (150mM NaCl, 50mM Tris HCl, 2mM EDTA, 50 μ M Triton X-100), 72 μ g 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 μ g leupeptin, 1 μ g aprotinin). Lysates were sonicated and centrifuged at 14,000 g for 3 minutes at 4°C. The supernatant from each sample was retained and stored at -70°C. A Bradford assay [39] was carried out to determine the protein concentration of the samples. The protein concentration of each sample was adjusted to 2mg/ml with distilled water and equal volumes of 2X Laemmli buffer (2% (w/v) SDS, 10% (v/v) mercaptoethanol, 20% (v/v) glycerol, 10% (w/v) Tris HCl (5mM, pH 6.8), 0.05% (w/v) bromophenol blue) was added. Samples were boiled and proteins separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Watford, UK) and probed with a rabbit polyclonal primary antibody against heme oxygenase-1, (1:250 dilution; kindly donated by Professor Tetsuro Ishii). A horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10000 dilution; Pierce Biotechnology, Rockford, IL, USA) was used with enhanced chemiluminescence (ECL; Amersham Biosciences, Little Chalfont, UK) to visualise the band on autoradiographic film (Amersham Biosciences). For comparison, membranes were also probed with a goat polyclonal antibody against the housekeeping protein α -actin (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

2.7. HPLC analysis of LDL lipids

Reverse-phase HPLC with 210nm and 234nm absorbance monitoring was

used to analyse the composition of each oxidised LDL species, based on methods described by Kritharides et al. [40]. LDL (3ml of 100µg protein/ml) was vortexed for 10 seconds with 3ml methanol, followed by addition of 3ml hexane and a 30 second vortex to transfer the more hydrophobic lipid products to the hexane fraction. The sample was centrifuged at 1500 g for 5 minutes at room temperature and 2.5ml of the top hexane fraction was removed and dried under nitrogen. The dried sample was redissolved in 200µl mobile phase before injection. HPLC analysis was performed using a Perkin-Elmer 200 series HPLC system. The sample (20µl) was injected on to a reverse-phase C-18 column (Phenomenex, Macclesfield, UK). Analysis of cholesteryl esters was performed by detection at 210nm after elution with acetonitrile/isopropanol/water (40/58.8/1.2, by vol.). Analysis of oxidised derivatives of cholesterol and cholesteryl esters was performed by detection at 234nm using a solvent of acetonitrile/isopropanol/water (44/52/4, by vol.). Quantification of cholesteryl esters and their oxidation products was performed using known concentrations of commercially available standards (Sigma-Aldrich).

2.8. HPLC analysis of intracellular ascorbate levels

Reverse-phase HPLC was used to analyse the intracellular concentration of ascorbate in J774 macrophages, based a method described by Wen et al.[41]. Briefly, after ascorbate (100µM) pretreatment, cotreatment or pre- plus cotreatment, or no ascorbate treatment, cells were exposed to moderately-oxidised LDL (4°C method; 200µg protein/ml) for up to 48h. Cells were washed three times with PBS and scraped in ice-cold 5% (w/v) metaphosphoric acid. An equal volume of 10% (w/v) metaphosphoric acid was then added to the samples. Each sample was vortexed and sonicated for 20 seconds. Samples were centrifuged at 14,000 g for 3 minutes and the supernatants collected and analysed immediately. HPLC analysis was performed on the supernatants (20µl) using a Perkin-Elmer 200 series HPLC system with a reverse-phase C-18 column. Analysis of

ascorbate was performed by detection at 230nm, after elution with KH_2PO_4 (25mM; pH 2.5). Ascorbate concentration was calculated using stock solutions of known concentration, also prepared in metaphosphoric acid.

2.9. Statistical analysis

Values are expressed as mean $\pm$ SE (where $n \geq 3$). Differences in mean values among data sets were determined using a paired or unpaired Student's *t* test, as appropriate. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Oxidised LDL species vary significantly in fatty acid composition

After noting in our earlier studies [14] that different oxidised LDL species varied in their cytotoxicity, their lipid composition was investigated by HPLC analysis. It was found that native and mildly-oxidised LDL contained low levels of the cytotoxic lipids cholesteryl linoleate hydroperoxide (CLOOH) and 7-ketocholesterol (Table 1). As LDL underwent oxidation, the levels of cholesteryl arachidonate and cholesteryl linoleate decreased and levels of CLOOH and 7-ketocholesterol increased. Moderately-oxidised LDL (37°C method) contained markedly elevated levels of CLOOH (73.3 ± 18.4 nmol/mg LDL protein; $P < 0.05$) compared to native LDL, and detectable levels of 7-ketocholesterol (13 ± 5 nmol/mg LDL protein; $P < 0.05$). Highly-oxidised LDL contained very low levels of CLOOH but a 10-fold increase in 7-ketocholesterol (134 ± 23 nmol/mg LDL protein; $P < 0.01$) compared to moderately-oxidised LDL. In addition, the relative electrophoretic mobility (REM) of the oxidised LDL species increased with the duration of oxidation. The REM of moderately-oxidised LDL (37°C method) and highly-oxidised LDL was significantly increased to 2.37 ($P < 0.01$) and 4.88 ($P < 0.01$) respectively (Table 1), reflecting the oxidative modification of the protein moiety of LDL and resulting in an increase in the net negative charge of the LDL particle.

A novel oxidised LDL species formed by dialysis at 4°C (moderately-oxidised LDL, 4°C method) contained a low level of 7-ketocholesterol but very high levels of CLOOH (149 ± 17 nmol/mg LDL protein; $P < 0.01$), and had a REM of 2.52. Due to its high toxicity (attributed to its elevated CLOOH content), simple preparation procedure, high yield and its broad similarity in composition to moderately-oxidised LDL produced at 37°C, this oxidised LDL species was used for all further experiments (with the exception

of experiments to determine the relative toxicities of the oxidised species prepared at 37°C; Figures 1 and 2).

3.2. Highly-oxidised LDL induces marked apoptosis of macrophages

To explore the relative toxicities of oxidised LDL species towards macrophages, J774 cells were incubated for 24 or 48h in DMEM with the addition of phosphate buffer, native LDL, or mildly-oxidised, moderately-oxidised (37°C method) or highly-oxidised LDL. LDL species were added at a concentration of 200µg protein/ml because preliminary studies indicated that this amount of highly oxidised LDL elicited a marked apoptotic response in J774 macrophages without increasing levels of primary necrosis. Toxicity was quantified by a MTT assay to detect a decrease in mitochondrial dehydrogenase activity, indicating cytotoxicity. Activity was significantly decreased after incubation with highly-oxidised LDL for 24h ($P<0.01$) or 48h ($P<0.001$), with a 69% fall at 48h (Figure 1). Incubation with moderately-oxidised LDL caused a significant decrease in activity after 48h ($P<0.01$), but much less than was observed with highly-oxidised LDL. Native LDL and mildly-oxidised LDL had no effect.

These findings were confirmed by flow cytometry using annexin V binding to detect phosphatidylserine externalisation, a specific marker for apoptosis. Incubation with either moderately-oxidised LDL (37°C method) or highly-oxidised LDL, compared to native LDL, significantly increased the percentage of annexin V-labelled cells after both 24h and 48h (Figure 2A). Highly-oxidised LDL was clearly the most toxic oxidised LDL species and induced phosphatidylserine externalisation in 62% of cells after 24h and in 86% after 48h. This is in contrast with our previous findings which showed moderately-oxidised LDL to be the most toxic species towards human umbilical artery smooth muscle cells [14]. This effect was not due to the difference in cell culture medium used in the

experiments, as when the experiments with J774 cells were repeated in MCDB131 medium (which was used for our previous study), similar results were obtained (data not shown).

Interestingly, highly-oxidised LDL was also shown to be the oxidised LDL species that induced the most phosphatidylserine externalisation in mouse peritoneal macrophages (Figure 2B) and in human monocytes (Figure 2C). However, moderately-oxidised LDL treatment significantly increased the levels of phosphatidylserine externalisation in mouse peritoneal macrophages, and both mildly- and moderately-oxidised LDL significantly increased phosphatidylserine externalisation in human monocytes.

3.3. Ascorbate augments macrophage apoptosis

Our previous study of apoptosis in smooth muscle cells from human umbilical arteries concentrated on the effects of moderately-oxidised LDL rich in LOOH [14]. We have therefore concentrated in the study reported here on the effects of moderately-oxidised LDL on apoptosis in macrophages. In the previous study, ascorbate (100 μ M) pretreatment for 24 hours of smooth muscle cells attenuated cytotoxicity induced by moderately-oxidised LDL, but in the present study ascorbate (100 μ M) treatment failed to afford protection toward J774 macrophages. Analysis of a late stage marker of apoptosis, DNA fragmentation, demonstrated that ascorbate failed to attenuate the apoptosis mediated by moderately-oxidised LDL (4°C method; Figure 3). Furthermore, DNA fragmentation was significantly increased at 24h by ascorbate (100 μ M) pretreatment ($P<0.01$) and by pre-plus cotreatment (where cells were pre-treated with ascorbate for 24h, then treated again with a fresh solution of ascorbate upon commencement of the experiment; $P<0.05$) and was significantly increased at 36h by all ascorbate treatments (ascorbate pretreatment $P<0.05$; ascorbate cotreatment $P<0.01$; ascorbate pre- plus cotreatment $P<0.05$). Although a trend towards a slight increase in fragmentation was noted at 48h, this increase was not

significant.

Analysis of annexin V binding confirmed that ascorbate pretreatment of J774 cells modestly, but significantly, increased the levels of phosphatidylserine externalisation induced by moderately-oxidised LDL (4°C method; Figure 4A). Cotreatment with moderately-oxidised LDL and ascorbate, and a combined ascorbate pre- and cotreatment also had this effect. Levels of apoptotic cells were consistently and significantly increased by 10-15% by the ascorbate treatments after 24h (all treatments $P<0.05$) and 5-10% after 48h (ascorbate pretreatment $P<0.001$; ascorbate cotreatment $P<0.05$; ascorbate pre- plus cotreatment $P<0.05$). Treatment of J774 macrophages with ascorbate alone did not significantly increase the percentage of apoptotic cells above the levels seen in control cultures (3.3% annexin V-positive cells in control cultures after 48h versus 3.6% in cultures treated with ascorbate (100 μ M) alone for 48h).

It has been suggested that the pro-apoptotic effect of ascorbate is an artefact of in vitro tissue culture experiments and is due to generation of cytotoxic concentrations of hydrogen peroxide in the culture medium on addition of ascorbate [42]. We have demonstrated however, that ascorbate still promoted moderately-oxidised LDL (4°C method)-induced apoptosis in the presence of catalase (750U/ml; Figure 4B). In addition, we have also demonstrated that ascorbate cotreatment modestly but significantly promoted moderately-oxidised LDL-induced apoptosis in human monocytes after 24h ($P<0.05$; Figure 4C). Again, treatment of human monocytes with ascorbate alone for 24h did not significantly increase the percentage of apoptotic cells above the levels found in control cultures (data not shown).

3.4. Intracellular ascorbate levels

We demonstrated using HPLC that the baseline intracellular level of ascorbate in J774 macrophages was low, on average 21.1 ± 4.1 nmol/mg cell protein (Figure 5). Cells treated with moderately-oxidised LDL ($200\mu\text{g}/\text{protein ml}$; 4°C method) alone maintained their low baseline level of ascorbate over 48h. Pretreatment with ascorbate (24h; $100\mu\text{M}$) greatly increased the intracellular ascorbate levels to 259 ± 30 nmol/mg cell protein, which fell to the baseline level by 24h, when they were exposed to moderately-oxidised LDL. Cells cotreated with ascorbate demonstrated increased intracellular levels by 6h (334 ± 21 nmol/mg cell protein), despite the addition of moderately-oxidised LDL, and maintained elevated levels throughout the treatment (172 ± 25 nmol/mg cell protein; 48h). Cells both pre- and cotreated with ascorbate had elevated intracellular levels on addition of moderately-oxidised LDL (237 ± 24 nmol/mg cell protein), which remained elevated throughout the treatment (241 ± 47 nmol/mg cell protein; 48h).

3.5. Ascorbate and oxidative stress

It has been shown previously that treatment of vascular cells with oxidised LDL can increase the expression of antioxidant stress proteins [30]. Here we demonstrate that as well as inducing apoptosis, moderately-oxidised LDL (4°C method) upregulated the expression of heme oxygenase-1 in a concentration-dependent manner (Figure 6). Interestingly, pretreatment with ascorbate ($100\mu\text{M}$; 24h) abrogated the increase of heme oxygenase-1 induced by moderately-oxidised LDL.

4. Discussion

Contrary to our previous findings using smooth muscle cells from human umbilical arteries [14], the study described here demonstrates that macrophages are more susceptible to apoptosis induced by a highly-oxidised LDL species rich in oxysterols than to a moderately-oxidised (37°C method) species rich in LOOH. Macrophages constitutively express the surface receptors CD36 [43], CD68 [44] and macrophage scavenger receptors AI/II [45], which bind and internalise oxidised LDL. Scavenger receptors may be expressed on intimal vascular smooth muscle cells of atherosclerotic lesions [46-48], but their expression is low in medial smooth muscle cells cultures [49]. Macrophages, therefore, have the capacity to internalise extensively oxidised LDL in copious quantities [50], which may explain why they are more sensitive to the apoptotic effects of highly-oxidised LDL. Our findings suggest that the type of cell most susceptible to the toxicity of oxidised LDL may depend upon the extent of oxidation of LDL in atherosclerotic lesions.

We have also noted in this study that in contrast to smooth muscle cells, ascorbate does not protect J774 macrophages or human monocytes against the cytotoxic effects of moderately-oxidised LDL (4°C method). Furthermore, ascorbate treatment was seen to modestly increase phosphatidylserine externalisation and DNA fragmentation. It is not unknown for ascorbate to display pro-apoptotic activity; ascorbate treatment induces apoptosis of human promyelocytic leukaemia HL-60 cells [51] and of the human salivary gland tumor HSG cell line [52]. Furthermore, ascorbate can promote the oxidation of LDL in vitro [53] and potentiated arsenic trioxide-induced apoptosis of peripheral blood mononuclear cells from patients with Sezary syndrome [54]. We examined the expression of the antioxidant stress protein heme oxygenase-1 as an indicator of the level of oxidative stress in oxidised LDL-treated macrophages. We and others have previously shown that oxidised LDL induced heme oxygenase-1 expression in macrophages in a concentration

dependent manner [29, 31, 55]. Treatment of J774 cells with moderately-oxidised LDL (4°C method) induced a concentration-dependent increase in heme oxygenase-1 protein expression, and this increase was prevented by ascorbate pre-treatment. Thus, at a concentration of 100µM, ascorbate simultaneously inhibited moderately-oxidised LDL-induced HO-1 expression (antioxidant behaviour), whilst promoting moderately-oxidised LDL-induced apoptosis (pro-apoptotic behaviour). Hence, the pro-apoptotic effect of ascorbate is unlikely to be mediated by a pro-oxidant mechanism, as cells treated with ascorbate and moderately-oxidised LDL underwent apoptosis despite apparent low internal levels of oxidative stress.

Little work has been done to elucidate the mechanisms behind the pro-apoptotic effects of ascorbate. It has been demonstrated that the apoptosis-inducing activity of ascorbate in human myelogenous leukemic cell lines is abrogated by catalase, implicating the involvement of hydrogen peroxide [56]. However, studies by Vissers et al. [57] in human umbilical vein endothelial cells treated with chlorinated oxidants showed that although ascorbate (1mM) was ineffective at preventing glutathione oxidation or protecting against the cytotoxicity of these agents, it appeared to protect the apoptotic machinery from oxidant damage, allowing progression of apoptosis over necrosis. Here, we have demonstrated a similar but more modest effect in macrophages, even in the presence of catalase and using a 10-fold lower and more physiologically relevant concentration [58] of ascorbate. We therefore propose that the pro-apoptotic effect of ascorbate observed in this study is due to the ability of ascorbate to protect components of the apoptotic machinery against oxidative insult.

Sustained oxidative stress can lead to caspase-3 inactivation [59], and it is likely that cells in atherosclerotic lesions are exposed to sustained levels of oxidative stress. It is

therefore reasonable to speculate that apoptosis in lesions may be perturbed, allowing cell death by necrosis to occur, thus propagating local inflammation. There is evidence to suggest that atherosclerotic lesions contain relatively high concentrations of ascorbate [60] and that phagocytes accumulate high internal ascorbate concentrations [58], possibly as protection against the oxidants they produce. Our finding that ascorbate may promote oxidised LDL-induced apoptosis in macrophages may therefore be beneficial in slowing the progression of atherosclerosis. If macrophage cell death is inevitable, apoptosis would be the favoured mechanism, as it would prevent cell lysis and subsequent release of pro-inflammatory lipids, cytokines and oxidative products into the local environment.

The mechanisms by which oxidised LDL induces apoptosis have yet to be fully elucidated. We have observed that treatment of J774 macrophages with moderately-oxidised LDL (4°C method) did not have a consistent effect on the protein levels of p53, PPAR γ or I κ B α (data not shown), suggesting limited involvement of these molecules in apoptosis induced by this type of oxidised LDL in J774 cells.

Ascorbate has been shown sometimes, but not always [23], to be beneficial in improving the outcome of various medical conditions, for example administration of ascorbate reversed vascular dysfunction in patients with coronary artery disease [61] and reduced plasma levels of circulating apoptotic microparticles in congestive heart failure patients [62]. Furthermore, ascorbate deficiency makes atherosclerotic plaques in mice less stable [19] and neutrophils from ascorbate-deficient mice are less likely to undergo either spontaneous or oxidant-induced apoptosis [63]. Although at first sight, our data may suggest that ascorbate may exacerbate the development of atherosclerosis by promoting apoptosis, if ascorbate is acting to decrease necrotic cell death, it may be of benefit in limiting the severity of the local inflammation in atherosclerotic lesions and slow the

progression of the disease.

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References

- [1] R. Ross, *Nature*. 362 (1993) 801-809.
- [2] M.R. Bennett, G.I. Evan, S.M. Schwartz, *J Clin. Invest.* 95 (1995) 2266-2274.
- [3] J.M. Isner, M. Kearney, S. Bortman, J. Passeri, *Circulation*. 91 (1995) 2703-2711.
- [4] S. Bjorkerud, B. Bjorkerud, *Am. J Pathol.* 149 (1996) 367-380.
- [5] Z. Mallat, A. Tedgui, *Br. J Pharmacol.* 130 (2000) 947-962.
- [6] Z. Mallat, B. Hugel, J. Ohan, G. Leseche, J.M. Freyssinet, A. Tedgui, *Circulation*. 99 (1999) 348-353.
- [7] D. Steinberg, *J Biol. Chem.* 272 (1997) 20963-20966.
- [8] Y. Okura, M. Brink, H. Itabe, K.J. Scheidegger, A. Kalangos, P. Delafontaine, *Circulation*. 102 (2000) 2680-2686.
- [9] B. Bjorkerud, S. Bjorkerud, *Arterioscler. Thromb. Vasc. Biol.* 16 (1996) 416-424.
- [10] V.C. Reid, S.J. Hardwick, M.J. Mitchinson, *FEBS Lett.* 332 (1993) 218-220.
- [11] M. Harada-Shiba, M. Kinoshita, H. Kamido, K. Shimokado, *J Biol. Chem.* 273 (1998) 9681-9687.
- [12] C.C. Hsieh, M.H. Yen, C.H. Yen, Y.T. Lau, *Cardiovasc. Res.* 49 (2001) 135-145.
- [13] J. Alcouffe, S. Caspar-Bauguil, V. Garcia, R. Salvayre, M. Thomsen, H. Benoist, *J Lipid Res.* 40 (1999) 1200-1210.
- [14] R.C. Siow, J.P. Richards, K.C. Pedley, D.S. Leake, G.E. Mann, *Arterioscler. Thromb. Vasc. Biol.* 19 (1999) 2387-2394.
- [15] J.R. Beetens, M.C. Coene, A. Verheyen, L. Zonnekeyn, A.G. Herman, *Biomed. Biochim. Acta.* 43 (1984) S273-276.
- [16] F.J. Finamore, R.P. Feldman, G.E. Cosgrove, *Int. J Vitam. Nutr. Res.* 46 (1976) 275-285.
- [17] A.J. Verlangieri, T.M. Hollis, R.O. Mumma, *Blood Vessels.* 14 (1977) 157-174.
- [18] D.W. Morel, M. de la Llera-Moya, K.E. Friday, *J Nutr.* 124 (1994) 2123-2130.

- [19] Y. Nakata, N. Maeda, *Circulation*. 105 (2002) 1485-1490.
- [20] J.E. Enstrom, L.E. Kanim, M.A. Klein, *Epidemiology*. 3 (1992) 194-202.
- [21] K.F. Gey, H.B. Stahelin, P. Puska, A. Evans, *Ann. N. Y. Acad. Sci.* 498 (1987) 110-123.
- [22] E.B. Rimm, M.J. Stampfer, A. Ascherio, E. Giovannucci, G.A. Colditz, W.C. Willett, *N. Engl. J Med.* 328 (1993) 1450-1456.
- [23] MRC/BHF Heart Protection Study, *Lancet*. 360 (2002) 23-33.
- [24] D. Li, T. Saldeen, J.L. Mehta, *Biochem. Biophys. Res. Commun.* 259 (1999) 157-161.
- [25] H. Schroeter, R.J. Williams, R. Matin, L. Iversen, C.A. Rice-Evans, *Free Radic. Biol. Med.* 29 (2000) 1222-1233.
- [26] C.E. Marchant, N.S. Law, C. van der Veen, S.J. Hardwick, K.L. Carpenter, M.J. Mitchinson, *FEBS Lett.* 358 (1995) 175-178.
- [27] R. Asmis, E.S. Wintergerst, *Eur. J Biochem.* 255 (1998) 147-155.
- [28] R.C. Siow, H. Sato, G.E. Mann, *Cardiovasc. Res.* 41 (1999) 385-394.
- [29] L.J. Wang, T.S. Lee, F.Y. Lee, R.C. Pai, L.Y. Chau, *Am. J Pathol.* 152 (1998) 711-720.
- [30] R.C. Siow, T. Ishii, H. Sato, S. Taketani, D.S. Leake, J.H. Sweiry, J.D. Pearson, S. Bannai, G.E. Mann, *FEBS Lett.* 368 (1995) 239-242.
- [31] M. Yamaguchi, H. Sato, S. Bannai, *Biochem. Biophys. Res. Commun.* 193 (1993) 1198-1201.
- [32] G.M. Wilkins, D.S. Leake, *Biochim. Biophys. Acta.* 1211 (1994) 69-78.
- [33] H. Esterbauer, G. Striegl, H. Puhl, M. Rotheneder, *Free Radic. Res. Commun.* 6 (1989) 67-75.
- [34] C. Rice-Evans, D. Leake, K.R. Bruckdorfer, A.T. Diplock, *Free Radic. Res.* 25 (1996) 285-311.

- [35] G.R. Schacterle, R.L. Pollack, *Anal. Biochem.* 51 (1973) 654-655.
- [36] M. el-Saadani, H. Esterbauer, M. el-Sayed, M. Goher, A.Y. Nassar, G. Jurgens, *J Lipid Res.* 30 (1989) 627-630.
- [37] Z.A. Cohn, B. Benson, *J Exp. Med.* 121 (1965) 153-170.
- [38] S.J. Martin, C.P. Reutelingsperger, A.J. McGahon, J.A. Rader, R.C. van Schie, D.M. LaFace, D.R. Green, *J Exp. Med.* 182 (1995) 1545-1556.
- [39] M.M. Bradford, *Anal. Biochem.* 72 (1976) 248-254.
- [40] L. Kritharides, W. Jessup, J. Gifford, R.T. Dean, *Anal. Biochem.* 213 (1993) 79-89.
- [41] Y. Wen, A. Sahni, C.A. Rea, X.H. Zhang, M.A. Khokher, B.M. Singh, *Diabetes Care.* 23 (2000) 254-255.
- [42] M.V. Clement, J. Ramalingam, L.H. Long, B. Halliwell, *Antioxid. Redox. Signal.* 3 (2001) 157-163.
- [43] G. Endemann, L.W. Stanton, K.S. Madden, C.M. Bryant, R.T. White, A.A. Protter, *J Biol. Chem.* 268 (1993) 11811-11816.
- [44] M.P. Ramprasad, W. Fischer, J.L. Witztum, G.R. Sambrano, O. Quehenberger, D. Steinberg, *Proc. Natl. Acad. Sci. U S A.* 92 (1995) 9580-9584.
- [45] T. Kodama, M. Freeman, L. Rohrer, J. Zabrecky, P. Matsudaira, M. Krieger, *Nature.* 343 (1990) 531-535.
- [46] R.E. Pitas, T.L. Innerarity, R.W. Mahley, *Arteriosclerosis.* 3 (1983) 2-12.
- [47] O. Jaakkola, O.P. Kallioniemi, T. Nikkari, *Atherosclerosis.* 69 (1988) 257-268.
- [48] T. Inaba, N. Yamada, T. Gotoda, H. Shimano, M. Shimada, K. Momomura, T. Kadowaki, K. Motoyoshi, T. Tsukada, N. Morisaki, *J Biol. Chem.* 267 (1992) 5693-5699.
- [49] R.E. Pitas, *J Biol. Chem.* 265 (1990) 12722-12727.
- [50] K.L. Carpenter, G.M. Wilkins, B. Fussell, J.A. Ballantine, S.E. Taylor, M.J. Mitchinson, D.S. Leake, *Biochem. J.* 304 (1994) 625-633.

- [51] K. Satoh, Y. Ida, M. Hosaka, H. Arakawa, M. Maeda, M. Ishihara, S. Kunii, Y. Kanda, M. Toguchi, H. Sakagami, *Anticancer. Res.* 18 (1998) 4371-4375.
- [52] K. Kusama, Y. Jiang, M. Toguchi, J. Ohno, H. Shikata, H. Sakashita, H. Sakagami, *Anticancer. Res.* 20 (2000) 151-154.
- [53] S.E. Stait, D.S. Leake, *Biochem. J.* 320 (1996) 373-381.
- [54] L. Michel, A. Dupuy, F. Jean-Louis, A. Sors, J. Poupon, M. Viguiet, P. Musette, L. Dubertret, L. Degos, H. Dombret, H. Bachelez, *J Invest. Dermatol.* 121 (2003) 881-893.
- [55] T. Ishii, K. Itoh, E. Ruiz, D.S. Leake, H. Unoki, M. Yamamoto, G.E. Mann, *Circ. Res.* 94 (2004) 609-616.
- [56] S. Fujinaga, H. Sakagami, N. Kuribayashi, H. Takahashi, Y. Amano, T. Sakagami, M. Takeda, *Showa Univ. J Med. Sci.* 6 (1994) 135-144.
- [57] M.C. Vissers, W.G. Lee, M.B. Hampton, *J Biol. Chem.* 276 (2001) 46835-46840.
- [58] M. Levine, C. Conry-Cantilena, Y. Wang, R.W. Welch, P.W. Washko, K.R. Dhariwal, J.B. Park, A. Lazarev, J.F. Graumlich, J. King, L.R. Cantilena, *Proc. Natl. Acad. Sci. U S A.* 93 (1996) 3704-3709.
- [59] S. Mohr, B. Zech, E.G. Lapetina, B. Brune, *Biochem. Biophys. Res. Commun.* 238 (1997) 387-391.
- [60] C. Suarna, R.T. Dean, J. May, R. Stocker, *Arterioscler. Thromb. Vasc. Biol.* 15 (1995) 1616-1624.
- [61] G.N. Levine, B. Frei, S.N. Koulouris, M.D. Gerhard, J.F. Keaney, Jr., J.A. Vita, *Circulation.* 93 (1996) 1107-1113.
- [62] L. Rossig, J. Hoffmann, B. Hugel, Z. Mallat, A. Haase, J.M. Freyssinet, A. Tedgui, A. Aicher, A.M. Zeiher, S. Dimmeler, *Circulation.* 104 (2001) 2182-2187.
- [63] M.C. Vissers, M.B. Hampton, *Biochem. Soc. Trans.* 32 (2004) 499-501.

Legends to Tables

Table 1. Lipid components (nmol/mg LDL protein) and relative electrophoretic mobility (REM) of native LDL and mildly-, moderately-, and highly-oxidised LDL species. Values shown are mean $\pm$ SE (n= 4-8 independent experiments). *P<0.05; **P<0.01; ***P<0.001, relative to native LDL.

Legends to figures

Fig. 1. Effects of native LDL and oxidised LDL species on mitochondrial dehydrogenase activity of J774 macrophages.

Cells were exposed to either phosphate buffer (control), native LDL, or mildly-, moderately- (37°C method) or highly-oxidised LDL (200µg protein/ml) for 24h (open bars) or 48h (shaded bars). Mitochondrial dehydrogenase activity was assessed by MTT assay and expressed as a percentage of the control absorbance at 540nm at the corresponding time point. Values are mean $\pm$ SE (n=4 independent experiments). **P<0.01; ***P<0.001, relative to control cells (unpaired Student's *t* test).

Fig. 2. Effects of native LDL and oxidised LDL species on phosphatidylserine externalisation.

[A] J774 macrophages were exposed to phosphate buffer (control), native LDL, or mildly-, moderately- (37°C method) or highly-oxidised LDL (200µg protein/ml) for 24h (open bars) or 48h (shaded bars). Phosphatidylserine externalisation was measured by annexin V labelling. Values are mean $\pm$ SE (n=4 independent experiments). **P<0.01; ***P<0.001 relative to control cells (paired Student's *t* test). [B] Mouse peritoneal macrophages were exposed to phosphate buffer (control), native LDL, or mildly-, moderately- (37°C method) or highly-oxidised LDL (200µg protein/ml) for 24h (open bars) or 48h (shaded bars). Values are mean $\pm$ SE (n=3 independent experiments). *P<0.05 relative to control cells (paired Student's *t* test). [C] Human monocytes were exposed to phosphate buffer (control), native LDL, or mildly-, moderately- (37°C method) or highly-oxidised LDL (200µg protein/ml) for 24h. Values are mean $\pm$ SE (n=4 independent experiments). *P<0.05 relative to control cells (paired Student's *t* test).

Fig. 3. Ascorbate increases moderately-oxidised LDL-induced DNA fragmentation.

After ascorbate (100 μ M) pretreatment, cotreatment or both pre- and cotreatment, or no ascorbate treatment, cells were exposed to phosphate buffer (control) or moderately-oxidised (mod-ox; 4°C method) LDL (200 μ g protein/ml) for 24h (open bars), 36h (hatched bars) or 48h (shaded bars). The extent of DNA fragmentation was determined by ELISA and is expressed as a percentage of the control absorbance at 450nm at 24h. Values are mean $\pm$ SE (n=4 independent experiments). *P<0.05; **P<0.01 relative to cells treated with moderately-oxidised LDL alone (unpaired Student's *t* test).

Fig. 4. Ascorbate augments moderately-oxidised LDL-induced phosphatidylserine externalisation in J774 macrophages.

[A] After ascorbate (100 μ M) pretreatment, cotreatment or both pre- and cotreatment, or no ascorbate treatment, cells were exposed to phosphate buffer (control) or moderately-oxidised (mod-ox; 4°C method) LDL (200 μ g protein/ml) for 24h (open bars) or 48h (shaded bars). Phosphatidylserine externalisation was measured by annexin V labelling. Values are mean $\pm$ SE (n=10 independent experiments). *P<0.05; ***P<0.001 relative to cells treated with moderately-oxidised LDL alone (paired Student's *t* test). [B] Ascorbate augments moderately-oxidised LDL-induced phosphatidylserine externalisation in the presence of catalase. The above experiment was repeated in the presence of catalase (750U/ml). Cells were treated with moderately-oxidised LDL (mod-ox; 4°C method) and ascorbate in the presence of catalase for 24h (open bars) or 48h (shaded bars). Phosphatidylserine externalisation was quantified by the extent of annexin V binding, as detected by flow cytometry. Values are mean $\pm$ SE (n=4 independent experiments). *P<0.05; **P<0.01 relative to cells treated with moderately-oxidised LDL alone (paired Student's *t* test). [C] Ascorbate augments moderately-oxidised LDL-induced phosphatidylserine externalisation in human monocytes. After ascorbate (100 μ M)

cotreatment, or no ascorbate treatment, cells were exposed to phosphate buffer (control) or moderately-oxidised (mod-ox; 4°C method) LDL (200µg protein/ml) for 24h. Phosphatidylserine externalisation was quantified annexin V labelling. Values are mean $\pm$ SE (n=4 independent experiments). *P<0.05 relative to cells treated with moderately-oxidised LDL alone (paired Student's *t* test).

Fig. 5. HPLC analysis of intracellular ascorbate concentration. After ascorbate (100µM) pretreatment, cotreatment or both pre- and cotreatment, or no ascorbate treatment, cells were exposed to moderately-oxidised (mod-ox; 4°C method) LDL (200µg protein/ml) for up to 48h. Cells were collected at selected time points and intracellular ascorbate concentration was measured by HPLC analysis. $\diamond$ represents treatment with mod-ox LDL alone; $\square$ represents ascorbate pretreatment (24h) followed by treatment with mod-ox LDL; Δ represents cotreatment with both ascorbate and mod-ox LDL; $\times$ represents ascorbate pretreatment (24h) followed by cotreatment with both ascorbate and mod ox LDL. Values are mean $\pm$ SE (n=4 independent experiments)

Fig. 6. Effect of ascorbate on heme oxygenase-1 expression. Cells were pretreated in the absence or presence of ascorbate (100µM; 24h) before exposure to phosphate buffer (control), native LDL (300µg protein/ml) or moderately-oxidised LDL (4°C method; 50-300µg protein/ml) for 24h, in the absence of ascorbate. Heme oxygenase-1 and α -actin expression was determined by Western blot analysis. Results are representative of data obtained from 4 independent experiments.