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Oxidation of Low-density Lipoprotein by Human Monocyte-Macrophages Results in Toxicity to the Oxidising Culture

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Human monocyte-macrophage cultures were exposed to native low density lipoprotein (LDL) for up to 24 h in Ham's F10 medium and the extent of cell-mediated LDL oxidation was determined by measurement of electrophoretic mobility on agarose gels and measurement of lipids and oxidised lipids (including 7β-hydroxycholesterol) by GC. After an initial lag phase, which varied from 2–8 h, there was a steady increase in oxidation over 24 h. No-cell control incubations showed minimal increases in oxidation over 24 h. Significant toxicity, measured as release of radioactivity from macrophages pre-loaded with tritiated adenine, was observed in the cells when they oxidised LDL and the extent of radioactivity release correlated closely with the extent of LDL oxidation. Inhibition of oxidation using α-tocopherol or probucol reduced toxicity within the oxidising culture. This self-inflicted toxicity may help to explain the origin and enlargement of the lipid core of advanced atherosclerotic lesions.

Key words: Low density lipoprotein; Oxidation; Monocyte-macrophages (human); Cytotoxicity, Antioxidants; Atherosclerosis

Abbreviations: EDTA, ethylenediaminetetraacetic acid; GC, gas chromatography; Ham's F10+, Ham's F10 medium plus phenol red, glutamine and iron; HMM, human monocyte-macrophages; MPM, mouse peritoneal macrophages; LDL, low density lipoprotein; PBS, phosphate-buffered saline; REM, relative electrophoretic mobility; 7β-OH, 7β-hydroxycholesterol; 18:2, linoleate; 20:4, arachidonate.

INTRODUCTION

The origin of the advanced atherosclerotic lesion, characteristic of industrialised populations, depends upon the development of the acellular lipid core.[1] How the fatty streak, composed of macrophage foam cells, gives rise to this core has been called the 'missing link' in atherogenesis.[2] There is now conclusive evidence that the death of macrophage foam cells is an important source of this acellular deposit.[3]

The cause of macrophage death in the lesion is unknown, but it was suggested some years ago that oxidation of lipids by the macrophages themselves might be responsible.[4] The toxicity of oxidised low density lipoprotein (LDL) for various

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cell-types *in vitro* was discovered by Chisolm and his colleagues,[5–7] who also showed that several cell-types, including macrophages,[7] were capable of causing LDL oxidation *in vitro*. The finding that oxidised LDL is toxic for macrophages *in vitro*,[8–12] including for a human monocytic cell-line (THP-1)[11] and for human monocyte-macrophages,[12] is much more recent.

The question that remains is whether macrophages in the lesion are capable of oxidising LDL sufficiently to cause self-inflicted toxicity. This appears to occur in mouse peritoneal macrophages *in vitro*,[8] but human monocyte-macrophages (HMM) have not been tested. We have therefore exposed HMM to native LDL *in vitro*, monitored their ability to oxidise the LDL and evaluated the resulting toxicity.

MATERIALS AND METHODS

All biochemicals were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K) unless otherwise stated and were of the highest purity available. [8-^{3}H]-Adenine (24 Ci/mmol) was obtained from Amersham Radiochemicals Ltd. (Aylesbury, Bucks, U.K.)

Culture of human monocyte-macrophages

Human monocyte-macrophages were isolated from the blood of adult volunteers using a method based on that of Cathcart and colleagues.[7] 120 ml of blood was obtained by venepuncture, EDTA (2.7 mM) added and the sample was centrifuged at 1500 *g*. The plasma was removed and replaced with phosphate buffered saline (PBS). The resulting cell suspension (30 ml) was then layered on to 15 ml Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and centrifuged at 600 *g* for 30 min. Mixed mononuclear cells were removed from the interface and washed three times by centrifugation with PBS containing bovine serum albumin (4 mg/ml).

For 12-well plate cultures, used for determination of oxidation by gas chromatography (GC) and relative electrophoretic mobility (REM), the cells were diluted to approximately 6×10^6 cells/ml in Iscove's Modified Dulbecco's medium (Gibco, Paisley, UK). 1 ml of this cell suspension was added to each well of Costar 12-well plates (Becton Dickinson, New Jersey, USA) which had been pre-coated with fetal calf serum (200 µl). After incubation for 1 h, the nonadherent cells were washed off with PBS, leaving a monolayer of monocyte-macrophages which were incubated in macrophage-SFM (Gibco) for up to 24 h before use. For cultures in 24-well plates (Falcon), used for toxicity assays and REM, 3×10^6 cells were seeded into each well in RPMI containing 10% LPD-FCS. This was replaced with the same medium following incubation for 1 h and washing off nonadherent cells with PBS.

The medium was replaced immediately before the addition of LDL and antioxidants with 1 ml Ham's F10 (ICN Biomedicals Inc. Costa Mesa, CA) supplemented with 3 µM freshly prepared $FeSO_4$ for 12-well plates or 7 µM for 24-well plates, 10.8 mg/l phenol red and 2 mM glutamine (Hams F10+) as used by Leake and Rankin,[13] thus bringing the concentration of $FeSO_4$ to 6 µM (12-well plates) or 10 µM (24-well plates) and the concentration of phenol red to 12 mg/l. The copper concentration in the medium was stated by the manufacturer to be 0.01 µM.

Preparation of LDL

LDL was prepared from pooled human EDTA-plasma from healthy volunteers by the method of Havel and colleagues[14] and stored at 4°C in 1 mM EDTA for up to 4 weeks before use. LDL was dialysed against PBS and diluted to 1 mg/ml. EDTA was added to give a final concentration in the medium of 10 µM for 12-well plates or 5 µM for 24-well plates before it was added to cells in culture wells. Probucol or DL-α-tocopherol dissolved in ethanol was also added to the wells at this stage if required, such that the final concentration of ethanol was 0.4% (v/v).

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Oxidation experiments

For GC experiments, 100 μg LDL was added to each well of a 12-well plate in a final volume of 1 ml Hams F10+, and incubated for up to 24 h. Control wells contained no cells. At each time-point, the medium from triplicate wells was harvested and pooled, centrifuged and dialysed against PBS containing 100 μM EDTA overnight until the phenol red was removed. 200 μM butylated hydroxytoluene was then added and the samples stored at –20°C until GC analysis was carried out. 50 μl samples were taken prior to freezing for measurement of REM and stored at 4°C.

Determination of electrophoretic mobility

Determination of electrophoretic mobility of samples was carried out by applying 5 μl of each medium sample to Paragon® LIPO lipoprotein electrophoresis gels (Beckman, Brea, California, USA). Gels were run at 100 V for 30 min, followed by fixing in a solution of ethanol, deionised water and glacial acetic acid (60:30:10 w/v), then drying and staining with Paragon® LIPO stain. Relative electrophoretic mobility was expressed as the distance travelled by the sample divided by the distance travelled by native LDL.

Extraction of lipids and gas chromatography

Lipids were extracted from the samples and processed for GC as described previously.[15] The procedure comprised addition of internal standards (n-heptadecanoic acid, coprostane and 5α-cholestane), Bligh and Dyer extraction, sodium borohydride reduction, saponification and derivatisation to methyl esters and trimethylsilyl ethers. Analysis of lipids using GC was performed as described previously[16] using a 30 m DB-1 fused silica capillary column, 0.32 mm internal diameter, 0.1 μm film thickness (J&W Scientific, Folsom, California, USA).

Toxicity assay

Cytotoxicity was determined in HMM pre-loaded with tritiated adenine as described previously.[17] Briefly, 0.5 μCi of tritiated adenine was added to each well of cells in a 24-well plate and incubated for 1 h. Unincorporated adenine was removed by washing with PBS and the medium replaced with Hams F10+. 100 μg of LDL was added to each well in a final volume of 0.5 ml Ham's F10+ and incubated for up to 24 h. The radioactivity in samples of medium and the intracellular radioactivity, from Triton X-100 (1% v/v) HMM lysates, were determined as disintegrations per minute on a liquid scintillation counter and the percentage release of radioactivity into the medium was calculated. Medium was taken from parallel cultures without [^{3}H]-adenine and stored at 4°C with 1 mM EDTA and 200 μM butylated hydroxytoluene for measurement of REM, to confirm that cell-mediated oxidation had occurred in each experiment.

RESULTS

The time-course of cell-mediated oxidation

LDL was exposed to HMM over 24 h and oxidation was assessed as relative electrophoretic mobility (REM) of the LDL in the medium compared to native LDL. There was a lag period, varying from 2 to 8 hours, during which no oxidation was detected (Figure 1a). After this, there was a rise in oxidation during up to 24 h culture, while the no-cells controls oxidised LDL to a lesser extent (Figure 1a). When incubation was extended to 48 h incubation, the level of oxidation had increased in the no-cells wells and reached the same level as in the cell cultures (data not shown).

GC analysis of the culture medium showed that accumulation of the oxidation product, 7β-hydroxycholesterol (Figure 1b) and decrease in concentrations of 18:2 fatty acid (linoleate) (Figure 1c) and 20:4 fatty acid (arachidonate) (Figure 1d) in the medium followed the same pattern as the increase in electrophoretic mobility. The correlations between REM and concentrations of 7β-hydroxycholesterol or 18:2 were linear (the former with a positive slope and the latter

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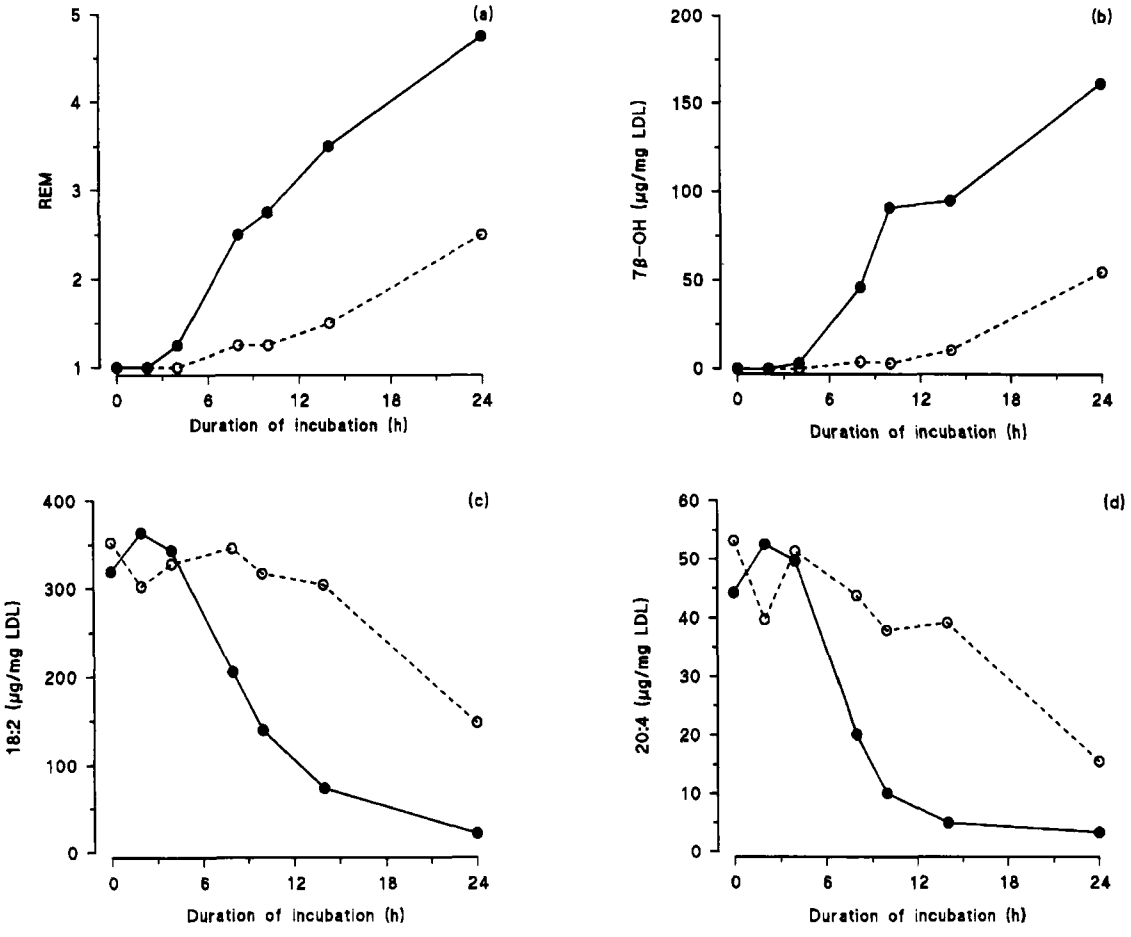

FIGURE 1 Oxidation of LDL by HMM over 24 h. LDL (100 μg/ml) was incubated with (●) or without (○) HMM in 12-well plates for up to 24 h. The medium was harvested and pooled from triplicate wells at each time point and the REM determined (a). The lipids were extracted and analysed by GC. The levels of 7β-hydroxycholesterol (b), linoleate (18:2) (c) and arachidonate (20:4) (d) are shown. Results are from one experiment which was representative of three separate experiments, each performed using HMM from different donors.

with a negative slope), and gave r^2 values of 0.97 and 0.94, respectively. The relationships between 18:2 or 20:4 and 7β-hydroxycholesterol were negatively sloped exponential curves with r^2 values of 0.95 and 0.96, respectively.

In some experiments in which the final concentration of $FeSO_4$ was 6 μM, cell-mediated oxidation of LDL did not occur at all and there was no toxicity in these cultures (data not shown). Increasing the final iron concentration in the medium to 10μM, by adding 7 μM fresh $FeSO_4$ in the subsequent toxicity experiments, ensured that HMM-mediated LDL oxidation occurred in every experiment, without significantly increasing oxidation within the cell-free controls.

The relationship between oxidation and toxicity

Further experiments looking at the time-course of HMM-mediated oxidation and toxicity within the oxidising culture showed an increase of toxicity over 24 h, measured as release of radioactivity from HMM pre-loaded with tritiated adenine (Figure 2a). This release was slower between 0 h and 8 h than at the later time-points, for the LDL-

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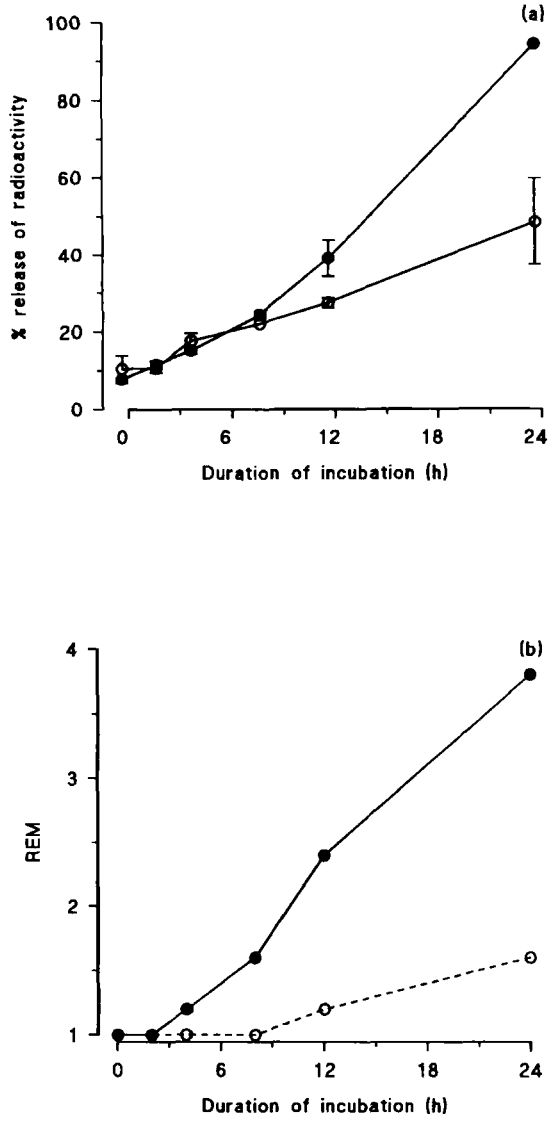

FIGURE 2 Toxicity in HMM cultured with LDL over 24 h and oxidation within the culture. HMM were pre-treated with tritiated adenine, then cultured with LDL (200 μg/ml;●) or without (○) LDL in 24-well plates. The percentage release of radioactivity into the medium was measured at each time point. Mean values and standard deviations from triplicate wells are shown (a). REM was measured in a fourth well, which had not been treated with tritiated adenine, containing HMM (●) and a cell-free control well (○) (b). Results shown are from one experiment which is representative of three separate experiments, each using HMM from different donors.

containing cultures. In the cultures without LDL, the level of radioactivity release from the cells was similar during the first 8 h, but thereafter significantly lower than in cultures with LDL. There was a similar trend of oxidation in parallel non-radioactive HMM cultures, measured by REM, and there was again a delay before cell-mediated oxidation could be measured (Figure 2b). Percentage release of radioactivity was plotted against REM for this experiment and two replicates. These gave good correlation (least squares linear regression) with r^2 values of 0.98, 0.98 and 0.89.

Inhibition of oxidation and toxicity by antioxidants

Concentrations of 8 and 80 μM probucol were used in this study. The higher concentration was similar to the average plasma levels in patients receiving a dose of 500 mg probucol twice daily (98μM).[18] The lower concentration was within the range of those previously used for inhibition of cell-mediated oxidation by probucol.[18,19] α-Tocopherol was used at concentrations which were equivalent to typical plasma concentrations found in supplemented individuals (80 μM).[20] A higher concentration (200 μM) was also used for maximal inhibition of oxidation.

Both the concentrations of the two antioxidants inhibited oxidation of LDL over 24 h when added to the cultures in ethanol, at the same time as the LDL. Cell-mediated oxidation was measured as REM (Figure 3a). Cytotoxicity was also diminished by the addition of either antioxidant (Figure 3b). Student's (paired) t-tests, performed on this data and data from two replicate experiments, showed that the inhibition of toxicity by the antioxidants was significant ($P<0.001$ for all concentrations of antioxidants, in three experiments, when compared to positive controls with LDL but no antioxidant added). The extent of toxicity in the presence of 80 μM probucol was not significantly different from that of a negative control with no LDL added.

We used two positive controls for cytotoxicity

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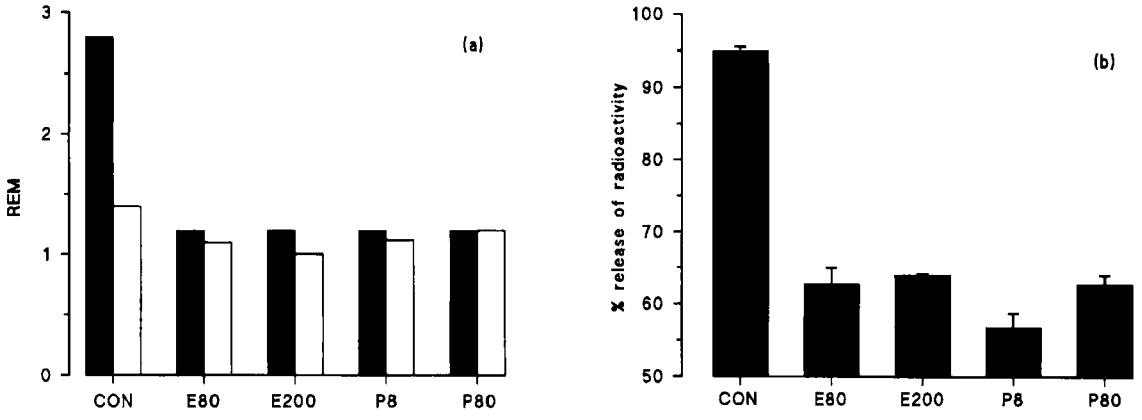

FIGURE 3 Inhibition of toxicity and oxidation by antioxidants. HMM cultures were pre-treated with tritiated adenine. LDL (200 μg/ml) plus 80 or 200 μM α-tocopherol (E80 and E200), 8 or 80 μM probucol (P8 and P80), was added to quadruplicate cultures in 24-well plates (one of which had not received tritiated adenine). The positive control had ethanol added but no antioxidant (CON). (a) REM was determined in the well containing HMM which was not radioactive (solid bars) and cell-free control wells (open bars) after 24 h. (b) Percentage release of radioactivity was measured after 24 h incubation. Bars represent the mean percentage release of radioactivity and error bars represent the standard deviations for the triplicate cultures. A control with no additions gave 55.8% release of radioactivity at 24 h. Results are from one experiment which was representative of three, performed using HMM from different donors.

in order to confirm that cytotoxicity within a culture did not itself initiate oxidation. One was menadione (2-methyl-1,4-napthoquinone), a compound which is capable of redox cycling and arylation and has been shown to be a potent cytotoxin in a variety of cell types.[21,22] The other was the calcium ionophore 4-bromo-A23187 which induces increases of intracellular free calcium ion, with resulting toxicity.[23] Menadione (200 μM) and 4-bromo-A23187 (2 μM) both induced near-maximal leakage of radioactivity (90.7% and 90.5%, respectively) from cells pre-loaded with tritiated adenine after 24 h exposure. However, these conditions did not produce any evidence of LDL oxidation, as the REM was equal to that of the cell-free control in both cases (data not shown).

DISCUSSION

Relevance to atherosclerosis

There is persuasive evidence that LDL oxidation occurs in the arterial wall during atherogenesis. There is depletion of polyunsaturated fatty acids in atherosclerotic lesions,[24,25] which also contain 7β-hydroxycholesterol, a product of free radical oxidation of cholesterol which is also produced by *in vitro* LDL oxidation, and 26-hydroxycholesterol, a product of oxidation of cholesterol by the cytochrome P450 sterol 26-hydroxylase.[25] Also the macrophages within the lesion contain ceroid, an insoluble complex of oxidised lipid and protein,[26] probably largely composed of oxidised LDL,[27] and react with antibodies raised against various forms of oxidised LDL.[28] Autoantibodies to oxidised LDL are found in the blood of many patients with advanced atherosclerosis.[29]

At present it is uncertain how the oxidation of LDL in the artery wall is initiated, as all cell types found in the lesion can oxidise LDL *in vitro*,[7,30–32] and auto-oxidation is also possible. However, the chemical evidence suggests that areas rich in foam cells are responsible for the most oxidative activity.[25]

The view that macrophage death is due to oxidation of LDL and is responsible for the development of the lipid core has recently received remarkably strong support from the finding that probucol prevents lipid core formation in Watanabe rabbits.[33]

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The time-course of cell-mediated LDL oxidation

The results show that there was a lag period before HMM-mediated LDL oxidation was detectable by the depletion of 18:2 and 20:4 fatty acids and production of 7β-hydroxycholesterol, measured by GC, and a similar lag period was observed for REM (Figure 1a–d). This is similar to the observations of Jessup and colleagues[34] when LDL oxidation mediated by mouse peritoneal macrophages (MPM) was measured by the increase in lipid hydroperoxides and degradation of LDL by target cells. Oxidation of LDL by copper ions leads to the generation of 7β-hydroxycholesterol[35] and depletion of 18:2 and 20:4 fatty acids after an initial lag period.[36] Carpenter and co-workers characterised the depletion of polyunsaturated fatty acids and generation of 7β-hydroxycholesterol in LDL oxidised by MPM.[16] The data from the present study show very similar trends.

The addition of freshly prepared $FeSO_4$ to the medium was required for HMM-mediated oxidation, as previously reported for MPM-mediated LDL oxidation.[16] However, in order to ensure that cell-mediated oxidation would occur in every experiment, a concentration of 7 μM added $FeSO_4$ was required rather than 3 μM, in the HMM cultures, indicating that the requirements of HMM are slightly different to those of MPM. The requirement for iron ions *in vitro* is not necessarily unnatural because the transition metals iron and copper have both been detected in human lesions, the iron in catalytic concentrations up to 7.2 μM.[37]

The variations in the duration of the lag period could be due to differences between donors in the oxidising capability of their HMM or in the content of natural antioxidants or polyunsaturated fatty acids of the different batches of LDL. Eventually, under the experimental conditions, LDL auto-oxidised in the absence of cells and, by 48 h incubation, oxidation in cell-free controls was similar to that obtained with cells (data not shown), indicating that the presence of cells only catalyses an event which occurs eventually in their absence.

The increase in REM and 7β-hydroxycholesterol and the depletion of linoleate correlated closely. There was also a good correlation between depletion of linoleate or arachidonate and increases in 7β-hydroxycholesterol. This indicates that all these changes occur in a concerted fashion during oxidation and measurement of any of these parameters apparently gives a good indication of the extent of LDL oxidation.

The relationship between oxidation and toxicity

The relationship between oxidation and toxicity is potentially important. Leake and colleagues remarked upon apparent cell damage within their oxidising cultures of MPM[13] but no quantitative data was presented. We have now measured toxicity within oxidising cultures of HMM preloaded with tritiated adenine, by quantifying the release of radioactivity (Figure 2a), and established the time-course of toxicity in cultures in relation to oxidation. Oxidation was confirmed in each experiment by measuring REM in parallel cultures (Figure 2b). Toxicity did not show a lag phase, as do our measurements of oxidation. It rose, although less steeply than later on, from the start. However, toxicity in the controls without LDL also increased in the same way over the first 8 h. Therefore if the control value is subtracted at each time point, a lag in toxicity is revealed (Figure 2a). We have thus demonstrated a close correlation between the extent of LDL oxidation by HMM and toxicity within the oxidising culture.

When an agent which is markedly toxic, such as menadione or calcium ionophore, is added to the cells at the start of the experiment, along with the LDL, there is no evidence of cell-mediated oxidation. This suggests that healthy cells are required to initiate oxidation and that toxicity is probably a result of the accumulation of toxic LDL oxidation products such as lipid peroxides and oxysterols. Various oxysterols have been shown repeatedly to be cytotoxic, including to HMM.[38]

The toxicity seen in the oxidising cultures

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might be due at least in part to lipid hydroperoxides, which are early products of LDL oxidation.[39] Toxicity increases more markedly in the later stages, suggesting that later stage oxidation products such as 7β-hydroxycholesterol might be major contributors.[38] Clues to the source of toxicity include the observation that cholesteryl linoleate is oxidised by MPM, with the production of 7β-hydroxycholesterol, and leads to toxicity, whereas cholesteryl oleate, with only one double bond, is neither oxidised nor leads to toxicity.[17]

Factors inhibiting oxidation and toxicity

No experiments *in vitro* can mimic exactly the events *in vivo*. In the atherosclerotic plaque, the cells are protected against oxidative damage both by their own defensive mechanisms and by exogenous antioxidants.

The cell's own defensive potential includes glutathione (GSH), which has been shown to increase in response to exposure to odised LDL.[40] Artificial depletion of GSH results in increased toxicity of oxidised LDL for THP-1 cells, a human monocytic cell line.[11] The present study did not address this important question of the role of cellular defences. These defences were clearly inadequate to prevent toxicity under the conditions used, but further experiments to address this question would be interesting.

Because in the present study the macrophage toxicity was apparently dependent on their capacity to oxidise the LDL, the effect of exogenous lipid-soluble antioxidants was examined. Addition of α-tocopherol or probucol simultaneously with the LDL caused an inhibition of LDL oxidation by the HMM (Figure 3a). Similar inhibition of oxidation by probucol has been observed in MPM cultures[41,42] and by α-tocopherol in endothelial cell cultures,[31] but the effect of antioxidants on toxicity have not previously been addressed in human cells. In the present study, inhibition of oxidation by the macrophages significantly reduced toxicity within the oxidising culture (Figure 3a and b), just as inhibition of copper-induced LDL oxidation by α-tocopherol diminishes its toxicity for HMM.[12]

If this information is interpreted in the context of the development of the atherosclerotic lesion, antioxidants could act by becoming incorporated into the LDL particle[38] and rendering LDL less susceptible to oxidation; by preventing LDL oxidation, they could prevent the toxicity towards macrophages and thus delay the onset of cell death within the lesion. The additional possibility, that exogenous antioxidants could make the cells somehow more resistant to damage by oxidation products, has been suggested by experiments in MPM. Pre-incubation of MPM with α-tocopherol for 24 h temporarily increased their resistance to a subsequent exposure to oxidised LDL.[8] Similar experiments in HMM, however, using pre-incubation for 24 h with doses of up to 200 êÌM α-tocopherol, have been entirely negative (data not shown).

In most studies on the toxicity of oxidised LDL to cells, toxicity has been measured in cultures of 'target' cells, to which previously-oxidised LDL is added. The present demonstration of toxicity within an oxidising culture raises the possibility that any cells, perhaps especially macrophages, which do oxidise LDL within the lesion may themselves become victims of the resulting toxic products.

Conclusion

In conclusion, human monocyte-macrophages cultured in Ham's F10 medium, supplemented with $FeSO_4$, oxidised LDL after an initial lag period. This oxidation resulted in cytotoxicity to the macrophages themselves and the extent of the toxicity correlated closely with the oxidation. The antioxidants α-tocopherol and probucol inhibited the cell-mediated LDL oxidation and therefore protected against cytotoxicity. These findings may help to explain the origin and growth of the lipid core of advanced atherosclerotic plaques.

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