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Cell-Mediated Oxidation of LDL: Comparison of Different Cell Types of the Atherosclerotic Lesion

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The three major cell types of the human atherosclerotic lesion – macrophages (Mø), smooth muscle cells (SMC) and endothelial cells (EC) – were compared for their ability to oxidise low density lipoprotein (LDL) *in vitro* under identical conditions. Near-confluent cultures were incubated for up to 48 h with 50 μg protein/ml LDL in Ham's F10 medium supplemented with 7 μM Fe^{2+}. All three cell types oxidised LDL readily using our culture conditions. After 24 and 48 h, the degree of LDL oxidation was in the order: Mø > SMC > EC when based on cell growth area and EC > SMC > Mø when based on cellular DNA content. However, LDL oxidation *in vitro* progressed more slowly between 24 and 48 h, probably due to increasing toxicity to the cells and/or depletion of polyunsaturated fatty acids. We therefore compared the time of onset of LDL oxidation. The earliest increase in LDL oxidation was always apparent with SMC. Gas chromatography revealed that LDL oxidation by all three cell types followed a similar pattern. The polyunsaturated fatty acids linoleic acid (18:2) and arachidonic acid (20:4) were depleted (to 10.3–18.1% and 4.5–24.7% respectively, compared to native LDL), whereas the content of stearic acid (18:0) and oleic acid (18:1) remained unchanged. Cholesterol was depleted (to 54.1–75.6% of native LDL) with a concomitant rise in 7β-hydroxycholesterol (to 60.6–128.1 μg/mg LDL). This corresponds to a conversion of 4.9, 9.5 and 10.4% of LDL cholesterol in EC-, SMC- and Mø-modified LDL respectively. All three cell types showed significant toxicity in the oxidising culture after 24 h. The possible relevance to LDL oxidation in atherosclerosis is discussed.

Keywords: Atherosclerosis, LDL oxidation, macrophages, endothelial cells, smooth muscle cells, cytotoxicity

INTRODUCTION

There is considerable evidence that oxidation of low density lipoprotein (LDL) is involved in atherogenesis.[1,2] Oxidised LDL (oxLDL) is present in atherosclerotic lesions[3] and antibodies to oxLDL are found in the serum of patients with advanced atherosclerosis.[4] Due to its chemotactic activity for human monocytes *in vitro*, oxLDL is thought to be partly responsible for the accumulation of monocyte-macrophages within the arterial lesion.[5] Further, oxLDL is avidly taken up by macrophages *in vitro*,[6,7] leading to intracellular accumulation of lipid[8] and ceroid.[9] Ceroid is an oxidation product of

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lipid–protein mixtures, resembles oxLDL[4,10] and is found *in vivo* in macrophage foam cells and within the lipid core of advanced plaques.[10–12] OxLDL is toxic to various cell types *in vitro*, including monocyte-macrophages,[13,14] vascular smooth muscle cells and endothelial cells.[15] The death of lipid-laden macrophage foam cells contributes to the enlargement of the lipid core during lesion progression.[16] Further evidence for the role of LDL oxidation in atherogenesis comes from the beneficial effect of antioxidants, such as probucol in experimental animals[17] and α-tocopherol in humans,[18] which appear to inhibit lesion progression.

The exact sites of LDL oxidation *in vivo* are still uncertain. As serum is a strong inhibitor of LDL oxidation,[19] the oxidation is generally thought to take place mainly within the arterial wall. We have taken the view that monocyte-macrophages are the most likely cell type to oxidise LDL *in vivo*.[4,9–12] However, monocyte-macrophages,[14,20] smooth muscle cells,[21] endothelial cells,[22] and lymphocytes[23] have all been reported to oxidise native LDL *in vitro* in the presence of available transition metals such as copper and iron, which are present in atherosclerotic plaques.[24–26] The direct comparison of these various studies is hindered by the use of cells from different sources and by varying experimental conditions. The question of which cell type in the lesion has the highest capacity for oxidation of LDL remains unanswered.

In this study therefore we directly compare mature human macrophages and subcultures of human aortic smooth muscle cells and human aortic endothelial cells for their capacity to oxidise LDL *in vitro* under identical experimental conditions.

MATERIALS AND METHODS

Chemicals

Organic solvents were purchased from Fisher (Leicester, UK) or BDH (Poole, Dorset, UK) and were of analytical grade or better. Chemicals and standards were from Sigma-Aldrich (Poole, Dorset, UK) and were of the highest purity available. Silylating reagent (*N,O*-bis(trimethylsilyl)-trifluoroacetamide plus 1% trimethylchlorosilane) was from Pierce and Warriner UK, Chester, UK.

Isolation of LDL

LDL samples were isolated from pooled human plasma of 5 or more healthy volunteers by ultracentrifugation in potassium bromide gradients according to Havel *et al.*[27] in the presence of 1 mM EDTA. Native LDL was dialysed extensively against 10 mM Tris/HCl buffer pH 7.4 containing 0.15 M NaCl and 1 mM EDTA, filter-sterilised (0.45 μm filters) and stored at 4°C for a maximum of 4 weeks. Protein concentrations of LDL were measured according to Lowry and colleagues[28] using bovine serum albumin (BSA) as standard. Prior to addition to the cells, LDL was dialysed for 24 h against phosphate-buffered saline (PBS) to remove EDTA and filter-sterilised.

Cell Culture

Mature human macrophages (Mø) were obtained by prolonged *in vitro* culture of human monocytes[29,30] isolated from HIV-screened human buffy coat (National Blood Transfusion Service, Brentwood, UK). Thirty millilitres of buffy coat was layered on to 15 ml of LymphoPrep (Nycomed Pharma AS, Oslo, Norway) and after centrifugation at room temperature for 30 min at 600× *g* the opaque interphase of mononuclear cells was removed and washed twice with PBS containing 4 mg/ml BSA to remove platelets. Then, monocytes were prepared by an additional centrifugation step in a Percoll gradient.[31] Mononuclear cells were resuspended in 4 ml of PBS and mixed with 8 ml of Percoll: Hanks' Balanced Salt Solution (10× concentrate) (6:1; at pH 7.0). After centrifugation at room temperature for 30 min at 400× *g*, the monocytes were

collected from the top of the gradient, washed twice in PBS/BSA and seeded in 24-well and 48-well tissue culture plates (Becton Dickinson, Franklin Lakes, New Jersey, USA) at a density of 5×10^5 and 2.5×10^5 cells/well, respectively. After incubation at 37°C for 1 h, any remaining non-adherent cells were removed by washing three times with PBS. Adherent monocytes were cultured in serum-free medium, Macrophage-SFM (Gibco, Paisley, Scotland, UK) for 10–14 days, renewing half of the culture medium in each well twice a week so as to minimise disturbance of cells. The purity of monocyte-macrophage preparations was examined after staining with the Simultest LeucoGATE kit (Becton Dickinson, San José, CA, USA) using LYSYS II software on a FACScan flow cytometer (Becton Dickinson). On average monocyte-macrophage purity was 90%.

Human aortic smooth muscle cells (SMC) were derived from normal aorta tissue by explant culture[32] and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% foetal calf serum, 2 mM L-glutamine, penicillin (63 µg/ml) and streptomycin (100 µg/ml). Alternatively, subcultures of human aortic SMC were purchased from TCS Biologicals Ltd. (Botolph Claydon, Buckingham, UK) at passage 3 and cultured in SmGM-2 medium (TCS Biologicals Ltd.) at a seeding density of 3500 cells/cm^2. Subcultures of human aortic endothelial cells (EC) were purchased from TCS Biologicals Ltd. at passage 3 and cultured in EGM medium (TCS Biologicals Ltd.) at a seeding density of 2500 cells/cm^2. Growth media were renewed every 2–3 days and cultures were used for experiments at 70–80% confluency to avoid cell overgrowth during the time course studies. SMC and EC were not used for experiments above passage 10 and 6, respectively.

Cell-mediated Oxidation of LDL

Unless stated otherwise, all subsequent experiments were performed in Ham's F10 medium (ICN Biomedicals Inc., Costa Mesa, CA, USA) supplemented with 2 mM L-glutamine and with 7 µM $FeSO_4$, thus bringing the total Fe^{2+} concentration to 10 µM.[14] The concentration of phenol red was 1.2 mg/l; the copper concentration was 0.01 µM as stated by the manufacturer. Cell monolayers were washed twice with PBS to remove the culture medium and native LDL was added at 50 µg protein/ml (1 ml/well). After 0, 2, 4, 8, 24 and 48 h, the supernatants of 2 identical wells were combined and 20 µM BHT (butylated hydroxytoluene, dissolved in ethanol) and 5 µM EDTA were added to prevent further oxidation. After centrifugation at $2000 \times g$ for 5 min to remove any floating cells, 1.4 ml of the supernatant was stored at −70°C until extraction and analysis by gas chromatography. The remaining supernatant was used immediately for the assessment of oxidation of LDL by agarose gel electrophoresis and by the TBA assay. As a measure of non-biological oxidation, identical LDL samples were incubated simultaneously under the same conditions in the absence of cells (NC-control).

Thiobarbituric Acid Assay

The formation of aldehydic peroxidation products in LDL was estimated by the thiobarbituric acid (TBA) assay using 1,1,3,3-tetramethoxypropane as standard[33] and Ham's F10 medium for all dilutions. Five hundred microlitres of culture supernatant was mixed with 250 µl of thiobarbituric acid (1.34% (w/v)) and 250 µl of trichloroacetic acid (40% (w/v)). After incubation at 90°C for 30 min, samples were spun at $15{,}000 \times g$ for 10 min and the absorbance of the supernatants was read at 532 nm. The degree of oxidation is expressed as nmoles malondialdehyde (MDA) equivalents/mg LDL or as nmoles MDA/(mg LDL × µg DNA).

Agarose Gel Electrophoresis

The electrophoretic mobility of LDL was examined by agarose gel electrophoresis on Paragon

'LlPO' gels (Beckman, Brea, CA, USA). Five microlitres of sample was applied to each lane and, after running the gels for 30 min at 100 V, gels were fixed for 5 min in ethanol : deionised water : glacial acetic acid (60 : 30 : 10 (v/v)), dried and stained with Paragon LIPOstain (Beckman, Brea, CA, USA). The relative electrophoretic mobility (REM) was calculated as the distance migrated by the sample divided by the distance migrated by native LDL.

Extraction and Work-up of Lipids

Lipids were extracted from the medium and processed for GC analysis as described previously.[34] The procedure comprised adding internal standards (n-heptadecanoic acid, 5α-cholestane and coprostane), Bligh and Dyer extraction (with sonication), sodium borohydride reduction, saponification and derivatisation to methyl esters and trimethylsilyl ethers. Processed samples were stored at −20°C under argon until GC analysis. Care was taken to minimise exposure of samples to air, and sodium borohydride reduction, saponification, derivatisation and storage of samples were all under nitrogen or argon.

Analysis by Gas Chromatography

After the above work-up GC analysis[34] was performed for all the samples, using a 30 m DB-1 fused silica capillary column (0.32 mm i.d., film thickness 0.1 μm) (J&W Scientific, Folsom, CA, USA) on a Carlo Erba Vega 6130 gas chromatograph equipped with an automated split-splitless injector operated in the splitless mode, and a flame ionisation detector. Samples were injected as hexane solutions. The column oven was temperature programmed at 50–120°C at 10°C/min, 120–200°C at 4°C/min, and 200–280°C at 3°C/min. Quantitation was by peak areas, measured electronically using a Carlo Erba DP8OO integrator, relative to the internal standards.

Measurement of DNA Content

Cellular DNA was measured using an established method.[35] After removing the culture supernatant for analysis of LDL oxidation, cells of two wells were mixed with 800 μl of DNA assay buffer (50 mM sodium phosphate, 2 M NaCl, 2 mM EDTA, pH 7.4). Any floating cells in the culture supernatant (see above) were pelleted by centrifugation, mixed with 200 μl of DNA assay buffer and combined with the cell sample of the wells. After sonicating on ice twice for 20 s (amplitude 22), 10 μl Hoechst dye 33258 was added to each sample to give a concentration of 1 μg/ml. The fluorescence was read on a luminescence spectrophotometer (Perkin Elmer Ltd., Beaconsfield, Bucks., UK) at excitation 356 nm/emission 458 nm. The DNA content of the samples was calculated by comparison with a standard curve (1–10 μg DNA/ml) prepared with calf thymus DNA (Sigma).

^{3}H-Adenine Assay

Toxicity was measured in 48-well plates using a tritiated adenine assay based on that of Andreoli and colleagues.[36] After washing twice with PBS, cells were loaded with 0.25 μCi (9.25 kBq) [8-^{3}H]adenine (Amersham Radiochemicals Ltd., Aylesbury, Bucks, UK) for 1 h at 37°C. Cells were rinsed three times with PBS and then incubated with native LDL for varying periods, as described above. Supernatants were removed, spun for 5 min at 1000 × g to remove any floating cells and the supernatant transferred to fresh tubes. Cells remaining in the wells were lysed in 500 μl Triton X-100 (1% (v/v)) and combined with the cell pellet of the centrifugation. Two hundred microlitres each of supernatant and lysate were mixed with 3 ml of OptiPhase 'Hi-Safe' scintillant (Fisons Chemicals, Loughborough, Leics., UK) and radioactivity was measured using a Packard Tricarb 1600 TR liquid scintillation analyser. The percentage of radioactivity leakage was calculated as: 100 × dpm supernatant/(dpm supernatant + cell lysate).

Statistics

The significance of results was calculated using the unpaired Student's *t*-test on Microsoft Excel 7.0. Curve fitting was performed using FigP software.

RESULTS

Choice of Oxidation Medium

The ability of cells to oxidise LDL depends on the composition of the cell culture medium used for the experiment.[37] We attempted initial oxidation experiments with mature human Mø, aortic SMC and EC using DMEM (Sigma) and RPMI-1640 (Sigma) cell culture medium in the absence of serum. No LDL oxidation was observed over the 48 h time course for any of the cell types using these media (data not shown). The lack of LDL oxidation in DMEM and RPMI has been reported previously[19,21,38] and is probably due to the extremely low concentration (0.25 μM $Fe(NO_3)_3$ in DMEM) or complete lack (in RPMI 1640) of added transition metals in these media.

Previous studies[14,19–21,38] have shown Ham's F10 medium to favour cell-mediated oxidation of LDL. Ham's F10 (ICN Biochemicals) contains 3 μM iron and 0.01 μM copper as stated by the manufacturer. In order to avoid oxidation being limited by insufficient supply of transition metals,[14] we supplemented this medium with an additional 7 μM $FeSO_4$. All oxidation experiments described below were performed using this medium. Results were compared either directly (cm^2 of confluent culture) or based on cellular DNA content. We found no significant differences in LDL oxidation between the SMC from different sources and the results were therefore combined.

Degree of Lipid Peroxidation

Aldehydic oxidation products generated in LDL in the presence and absence of cells were measured with the TBA assay. The content of TBA-reactive substances (TBARS) in LDL significantly increased during the incubation with all 3 cell types and in the absence of cells (Figure 1). After 48 h, the TBARS content averaged 32.95, 26.4 and 23.2 nmoles MDA/mg LDL for Mø-, SMC- and EC-modified LDL respectively and 6.65 nmoles MDA/mg LDL for LDL incubated in medium only (Figure 1A). A significant increase above NC-controls was seen

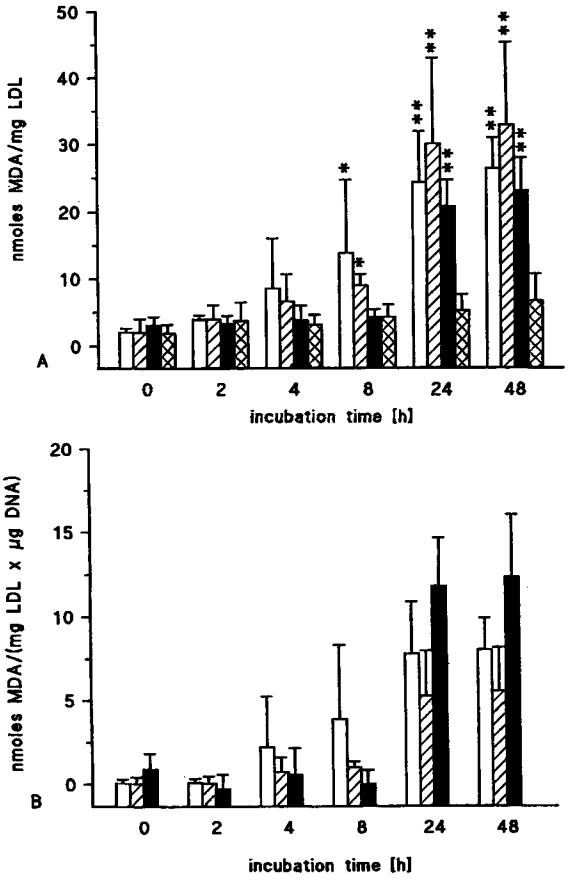

FIGURE 1 Lipid peroxidation of cell-oxidised LDL. LDL (50 μg/ml) was incubated with SMC (open bars), Mø (hatched bars), EC (solid bars) and in the absence of cells (NC-control, cross-hatched bars) over 48 h. The medium from duplicate wells was harvested and pooled at each time point and the degree of lipid peroxidation was measured with the TBA assay. Results represent the mean ± standard deviation of 3 to 5 separate experiments (* = $p \leq 0.05$, ** = $p \leq 0.001$ compared to NC-control) and are expressed in nmoles MDA/mg LDL in (A) and based on cellular DNA-content in (B).

after 8 h for SMC- and Mø-modified LDL and after 24 h also for EC-modified LDL. At 8 h, Mø-modified LDL was significantly more oxidised than EC-modified LDL ($p \leq 0.05$).

Oxidation experiments were performed with near-confluent cell cultures. However, EC and SMC are much larger in culture than Mø and therefore were less numerous in each well. In order to compare the oxidative capacity of equal numbers of cells, the DNA content of cell lysates was measured after the removal of supernatant LDL. No significant changes in DNA content for any of the cell types was observed over the 48 h time course. This is probably due to the fact that monocyte-derived Mø do not appear to proliferate under our culture conditions and SMC and EC were used for experiments at near-confluency. Oxidation results were therefore based on the average DNA content which were 2.46, 4.74 and 1.33 µg DNA/ml (or 4 cm^2, i.e. the area of 2 wells) for SMC, Mø and EC respectively. The average TBARS content after 48 h was 8.0, 5.55 and 12.4 nmoles MDA/(mg LDL × µg DNA) for SMC-, Mø- and EC-modified LDL, respectively (Figure 1B). The increased amounts of TBARS in LDL modified by EC compared to Mø were significant after 24 and 48 h ($p \leq 0.05$). Otherwise there were no significant differences between the three cell types.

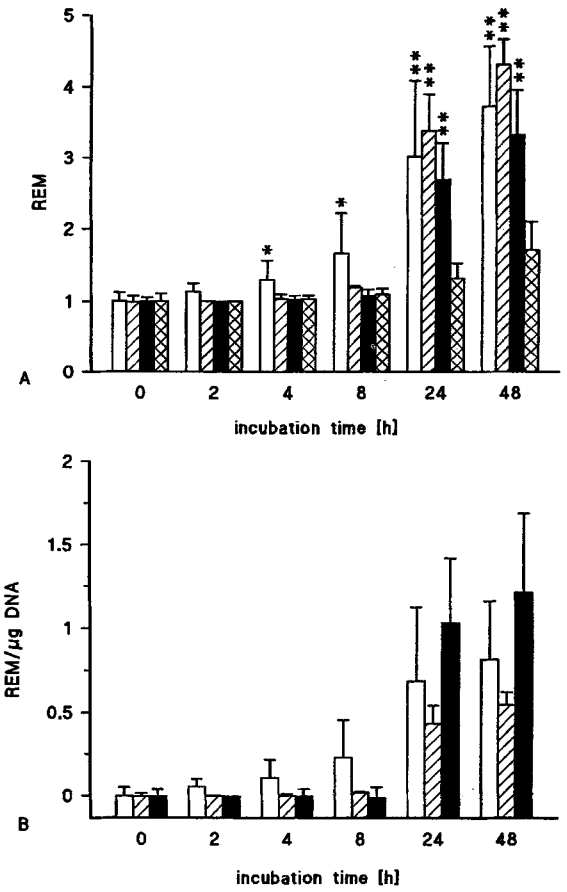

FIGURE 2 Electrophoretic mobility of cell-oxidised LDL. LDL (50 µg/ml) was incubated with SMC (open bars), Mø (hatched bars), EC (solid bars) and in the absence of cells (NC-control, cross-hatched bars) over 48 h. The medium from duplicate wells was harvested and pooled and the REM of cell-modified LDL determined. Results represent the mean ± standard deviation of 3 to 4 separate experiments (* = $p \leq 0.05$, ** = $p \leq 0.001$ compared to NC-control) and are based on cm^2 of confluent culture in (A) and on cellular DNA content in (B).

Electrophoretic Mobility of LDL

During LDL oxidation aldehydic products of lipid peroxidation react with the ε-amino group of lysine residues in apolipoprotein B100.[39] This reaction increases the electrophoretic mobility of LDL measured by agarose gel electrophoresis. For all three cell types and for the NC-control, the electrophoretic mobility of LDL increased with incubation time (Figure 2A). On average the relative electrophoretic mobility (REM) of LDL was 4.3, 3.7 and 3.3 after 48 h with Mø, SMC and EC, respectively and 1.7 in the absence of cells. Compared to NC-controls, the increase in REM became significant after 4 h with SMC, and after 24 h with Mø and EC. After 48 h, Mø-modified LDL was significantly ($p \leq 0.05$) more oxidised than LDL incubated with EC. Otherwise, there were no significant differences in the electrophoretic mobilities of cell-oxidised LDLs.

Based on the cellular DNA content, the increase in REM after 24 and 48 h was highest in EC-modified LDL, followed by SMC- and Mø-modified LDL (Figure 2B). The difference in REM

between EC- and Mø-modified LDL was significant at 24 and 48 h ($p \leq 0.05$).

GC-analysis of Oxidised LDL

Differences in the composition of LDL oxidised by the various cell types was analysed by GC. The contents of individual lipids and oxidised lipids presented below are the sum of their free and esterified forms in LDL and no differentiation of the degree of esterification was performed in this study. The results are given as percentage based on the 0 h-time point (100%) or in μg/mg LDL (Figure 3). As GC results based on DNA content were essentially the same, only the results based on cell growth area are presented.

Stearic Acid and Oleic Acid

The concentrations of stearic acid (18 : 0) and oleic acid (18 : 1) were 71.7 ± 25.1 μg/mg LDL and 222.8 ± 55.4 μg/mg LDL at the 0 h-time point respectively and were not significantly altered during the 48 h incubation with any of the cell types tested nor in the NC-control (Figure 3A and B).

Linoleic Acid and Arachidonic Acid

The concentration of linoleic acid (18 : 2) at the 0 h-time point was 392.65 ± 84.7 μg/mg LDL. After 48 h incubation with SMC, Mø and EC, the levels of linoleic acid in LDL fell to 11.3, 10.3 and 18.1%, respectively (Figure 3C and D). Compared to the NC-control (66.8% after 48 h), the decreases in linoleic acid concentration were significant for all three cell types after 24 and 48 h.

The content of arachidonic acid in LDL was 60.0 ± 14.8 μg/mg LDL at 0 h and appeared to decrease to 24.7%, 7.6% and 4.5% after 48 h incubation with SMC, Mø and EC, respectively. However, the decrease was only significant for EC-oxidised LDL after 24 and 48 h and for SMC-modified LDL after 8 h because the content of arachidonic acid in the NC-control also fell considerably due to non-biological oxidation (33.3% after 48 h).

Cholesterol

The concentration of cholesterol in LDL was 1282.1 ± 255.3 μg/mg at the 0 h-time point. During the 48 h incubation, cholesterol was moderately depleted and fell to 54.1%, 75.6% and 74.5% in SMC-, Mø- and EC-modified LDL, respectively (Figure 3E). Compared to the NC-control (89.9% after 48 h), the decreases were significant after 24 and 48 h for Mø and SMC and after 48 h for EC.

7β-Hydroxycholesterol

During the incubation with SMC, Mø and EC, the content of 7β-hydroxycholesterol (7β-OH-C) rose from nearly undetectable levels in unmodified LDL to concentrations of 117.1, 128.1 and 60.6 μg/mg LDL respectively after 48 h (Figure 3F). These increases were significant after 24 and 48 h, when compared to the NC-control (9.4 μg/mg LDL after 48 h).

Based on the cellular DNA content, the amount of 7β-OH-C formed after 48 h incubation with SMC, EC and Mø was 43.8, 38.5 and 25.0 μg/(mg LDL × μg DNA), respectively.

Cytotoxicity

Cell- and copper-oxidised LDL have previously been shown to be toxic to a number of cell types.[13,40,41] We used the tritiated adenine assay, which monitors the integrity of the plasma membrane, to measure the toxicity of LDL oxidation to the oxidising cultures of Mø, SMC and EC (Figure 4). Over the time course of 48 h, radioactivity leakage increased for all three cell types in the presence and absence of native LDL. However, the increases were higher in the presence of LDL than in its absence and became significant after 24 h. The radioactivity release after 48 h for Mø, SMC and EC was 47.0%, 61.9% and 73.1% in the absence and 71.45%, 87.5% and 90.0% in the presence of LDL, respectively. No significant differences in toxicity of LDL oxidation between the cell types were apparent.

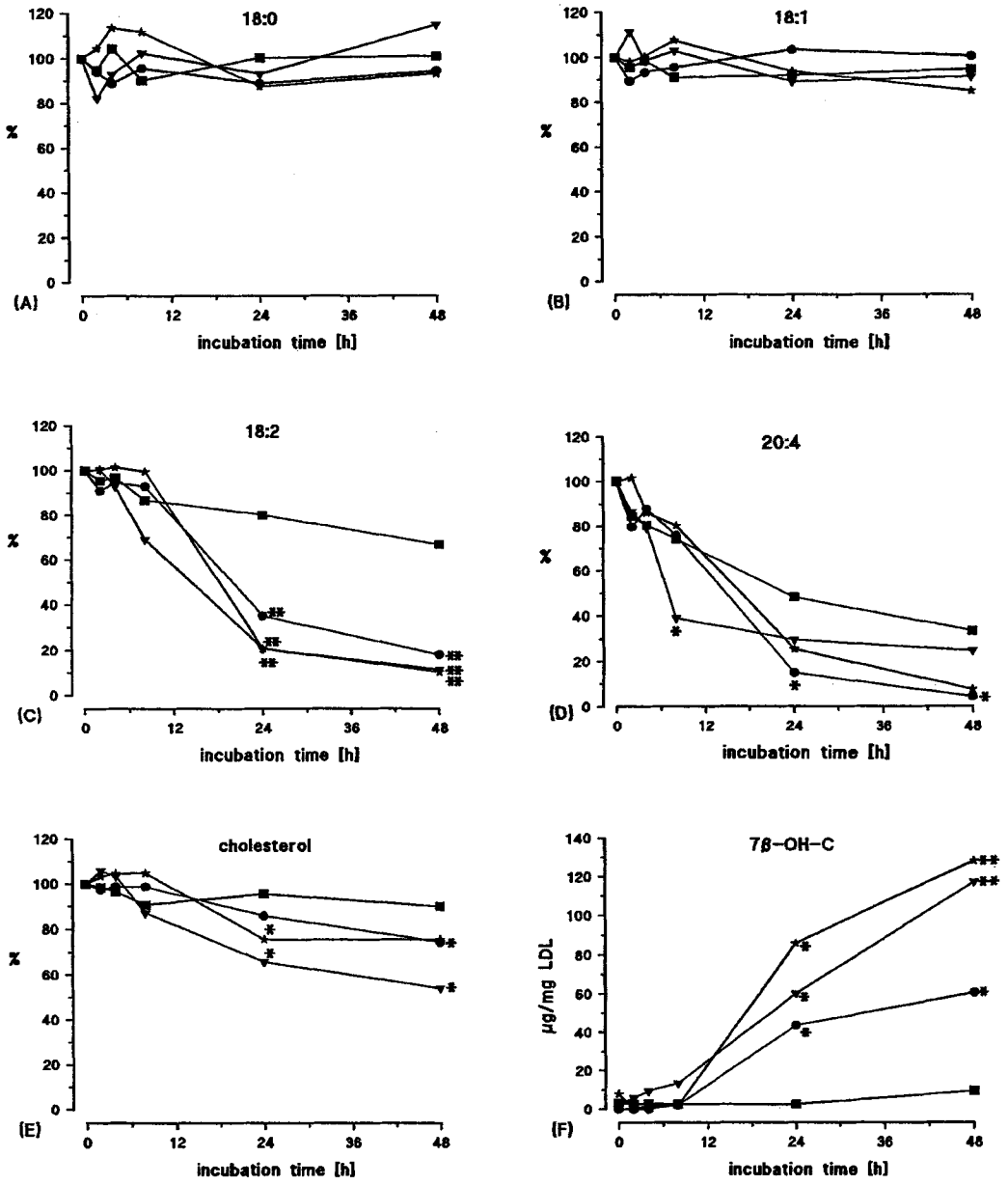

FIGURE 3 GC analysis of cell-oxidised LDL. LDL (50 µg/ml) was incubated with Mø (⋆), SMC (▼), EC (●) or in the absence of cells (■) over 48 h. The medium from duplicate wells was harvested and pooled at each time point, extracted and analysed by GC. With the exception of 7β-OH-C, which is in µg/mg LDL, the results are given as % based on the 0 h-time point (100%). The values represent the mean of 3 separate experiments (⋆ = $p \leq 0.05$, ⋆⋆ = $p \leq 0.001$ compared to the NC-control).

The radioactivity leakage observed in the absence of LDL is probably due to the lack of protein or serum during the incubation as a similar increase in leakage was observed after 48 h in serum-free RPMI 1640 and DMEM, especially for EC and SMC (data not shown). Radioactivity leakage in the absence of LDL was also observed in Ham's F10, but the addition of

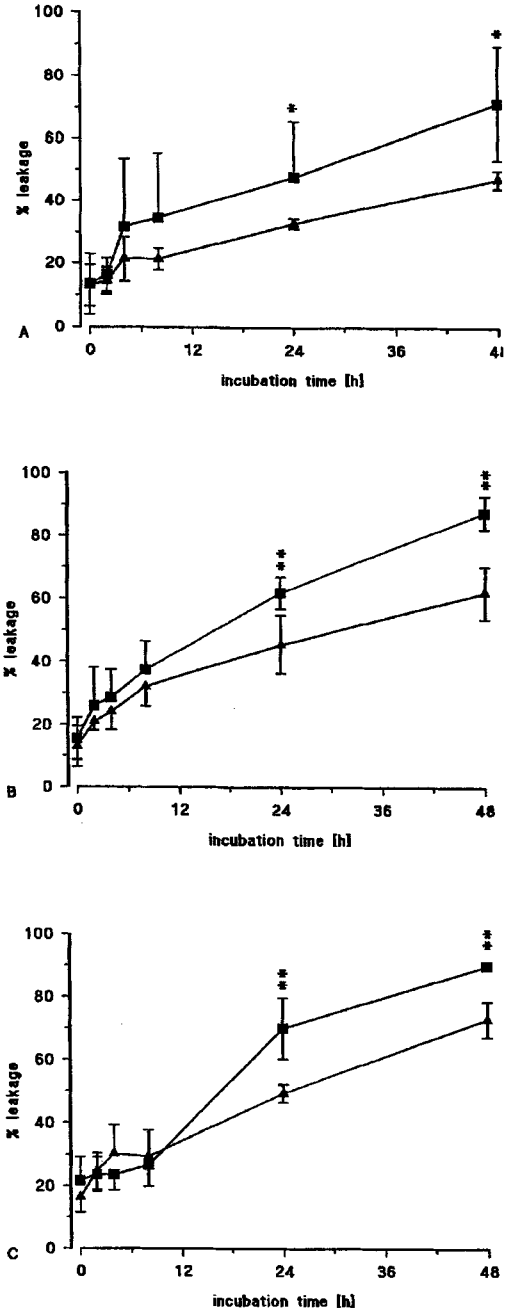

FIGURE 4 Toxicity of cell-oxidised LDL. Mø (A), SMC (B) and EC (C) were loaded for 1 h with tritiated adenine and subsequently incubated with 50 μg/ml LDL (■) or with medium only (▲) over 48 h. The medium was removed at each time point, the remaining cells were lysed and the radioactivity leakage was measured by liquid scintillation counting. The results represent the mean ± standard deviation of 3 to 4 separate experiments performed in triplicate (* = $p \leq 0.05$, ** = $p \leq 0.001$).

extra Fe^{2+} did not increase toxicity any further (data not shown).

Correlation Analysis

We used the values of Figures 1–4 for correlation plots, the results of which are summarised in Figure 5. The distribution of data points corresponding to Mø, SMC and EC within each graph was very similar and the regression curves were drawn, therefore, using the combined data. The depletion of linoleic acid (18:2) shows a strong, negative exponential correlation with TBARS (Figure 5A), electrophoretic mobility (Figure 5B) and formation of 7β-OH-C (Figure 5C). Electrophoretic mobility and formation of 7β-OH-C are positively correlated in a linear fashion (Figure 5D). Toxicity shows a moderate, positive logarithmic correlation with TBARS ($r^2 = 0.624$) and electrophoretic mobility ($r^2 = 0.676$). These cross-correlations show that the parameters we measured are associated, but not necessarily in a causal manner.

DISCUSSION

Mode of LDL Oxidation

All three cell types examined oxidised LDL in a similar fashion, apparently by means of free radicals. The polyunsaturated fatty acids linoleic acid (18:2) and arachidonic acid (20:4) were depleted during oxidation, but the levels of the saturated fatty acid, stearic acid (18:0) and the monounsaturated oleic acid (18:1) remained unchanged. Cholesterol levels fell moderately, concomitant with an increase in 7β-OH-C.

Oxidation probably starts with the polyunsaturated fatty acids, leading via a radical-mediated reaction to the formation of lipid hydroperoxides. These lipid hydroperoxides decompose to shorter-chain aldehydes[42] which react with the amino group of lysine residues in apo B-100.[39] Polyunsaturated fatty acid-derived radicals

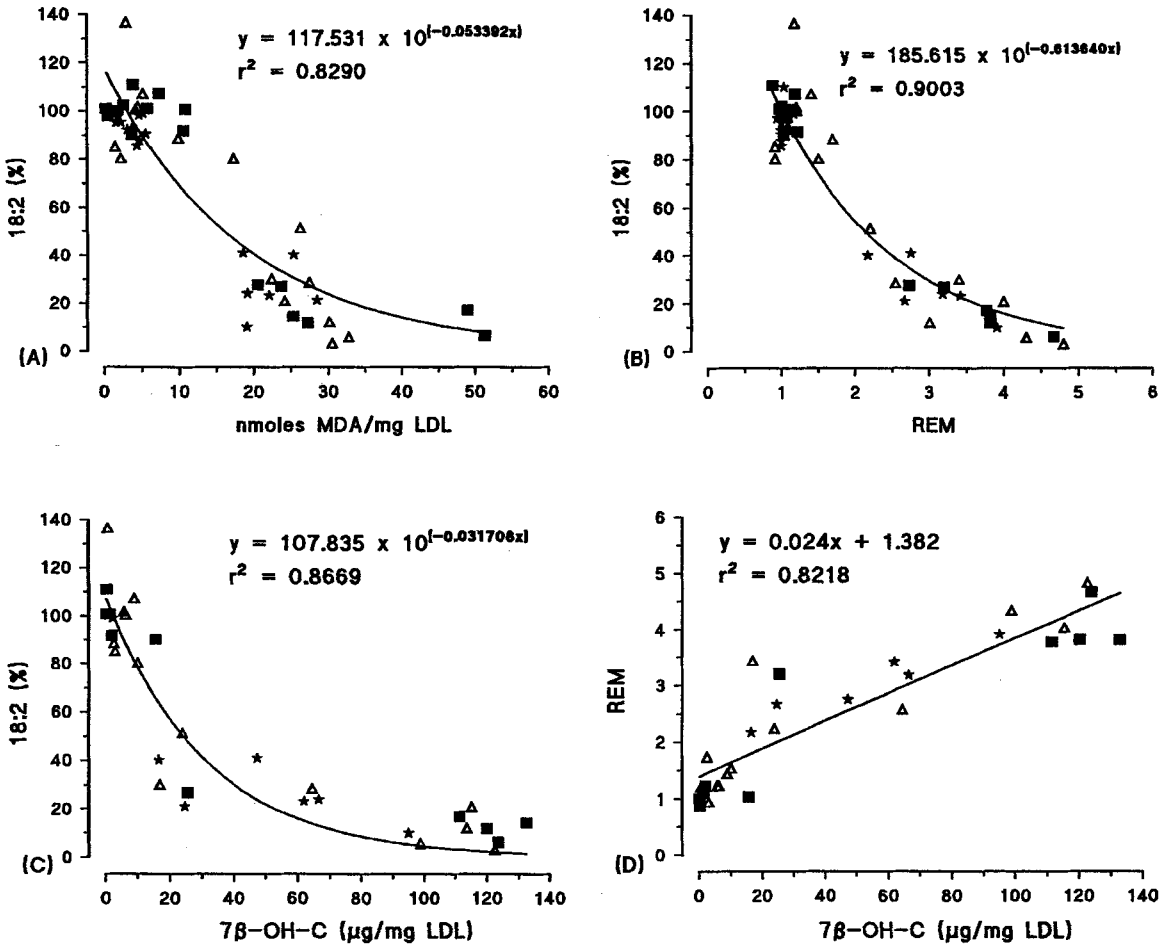

FIGURE 5 Correlation analysis using the data from Figures 1–4. As in previous figures, linoleic acid (18:2) is expressed in percent based on the 0 h-time point and 7β-OH-C is expressed as µg/mg LDL. Curve fitting was performed with FigP software using the combined data of all three cell types. Equations of fitted curves and correlation coefficients are given on each graph. (■) values for Mø, (△) values for SMC, (⋆) values for EC.

abstract hydrogen from the 7-position of cholesterol forming a resonance-stabilised allylic radical, which picks up oxygen leading to 7α- and 7β-hydroperoxy cholesterols, which subsequently give rise to 7α-hydroxy-, 7β-hydroxy- and 7-ketocholesterols.[34,43,44]

Similar patterns of radical-mediated LDL oxidation by human monocytes,[14] mouse peritoneal macrophages and Cu^{2+}[20] have been observed in previous studies. 26-Hydroxycholesterol (26-OH-C), found in lesions[45] and known to be produced *in vivo* by enzymatic oxidation of cholesterol,[46,47] was found in negligible amounts, as in our previous *in vitro* studies.[14,20]

The data support the view that radical-mediated LDL oxidation, once initiated, follows a consistent pattern, irrespective of the initiating event.[48] Different mechanisms may operate in different cell types, but all lead to peroxidation of polyunsaturated fatty acid chains. The close cross-correlation of the various parameters of oxidation strongly suggests that the end result is similar regardless of whether the oxidation is biologically or non-biologically initiated.[49]

Time Courses of Lipid Peroxidation and Electrophoretic Mobility

In agreement with other studies, no cell-mediated LDL oxidation was observed when experiments were carried out in media containing little or no transition metals, such as DMEM or RPMI.[19,21,38] In contrast, LDL was readily oxidised by all three cell types in Ham's F10 medium containing Fe^{2+} and Cu^{2+}, emphasising the dependence of cell-mediated LDL oxidation on the presence of these ions.[14,19,21] After an initial delay-phase during which no LDL oxidation was apparent, the content of lipid peroxidation products and the electrophoretic mobility of cell-oxidised LDL rose significantly above controls and then started to plateau between 24 and 48 h. Based on cell growth area, the order of oxidation after 24 and 48 h was Mø > SMC > EC, whereas based on DNA content, the order of oxidation was: EC > SMC > Mø. The lower rate of LDL oxidation after 24 h might be caused by decreased cell activity due to increasing toxicity, by depletion of polyunsaturated fatty acids in LDL, or both. Therefore, the degree of LDL modification after 24 and 48 h may not be an appropriate measure for the oxidative potency of cells. However, irrespective of whether LDL modification was based on cell growth area or on DNA content, the shortest delay-phase was always seen in the presence of SMC, followed by Mø and EC. Although the TBA assay seemed to indicate that Mø oxidise LDL more quickly than EC, an additional time point between 8 and 24 h would have been needed to differentiate clearly between the two cell types.

This delay in LDL oxidation by cells or Cu^{2+} has been observed previously[14,19,20,23,48,50] and its duration is affected by the LDL content of polyunsaturated fatty acids and of antioxidants, such as α-tocopherol and carotenoids.[48] Our oxidation experiments were performed with LDL derived from pooled serum samples of at least 5–6 subjects. Therefore, the variable delay-phase seen in the different cell types is less likely to be due to this variation between LDL batches than to different abilities of the cell types for LDL oxidation.

Cytotoxicity

All three cell types oxidising LDL showed similar evidence of toxicity after 24 and 48 h. LDL oxidised by cells and Cu^{2+} has previously been shown to be toxic to monocyte-macrophages,[13,14,40,41] smooth muscle cells and endothelial cells[15] and LDL oxidation by human monocytes results in toxicity to the oxidising culture.[14] Several compounds formed in LDL during oxidation are cytotoxic, including 4-hydroxynonenal[51] and oxysterols, such as 7-ketocholesterol, 7α- and 7β-hydroxycholesterol.[15,52]

7β-OH-C was formed during LDL peroxidation by monocytes[14] and mouse peritoneal macrophages[20] in previous studies and here by Mø, SMC and EC. The 7β-OH-C could arise at least in part from 7β-hydroperoxycholesterol or 7-ketocholesterol. 7β-Hydroperoxycholesterol has been identified in Cu^{2+}-oxidised LDL and is toxic to fibroblasts.[53] A previous study of Cu^{2+}- and macrophage-mediated LDL oxidation showed that approximately a quarter of the measured 7β-OH-C was derived from 7-ketocholesterol.[20] LDL oxidation also produces 7α-hydroxycholesterol at around one-third the level of 7β-OH-C,[20] but because 7α-hydroxycholesterol elutes close to the large cholesterol peak on GC, and in many cases is obscured by it, we did not attempt its quantitation. 26-OH-C, shown previously to be toxic to human monocyte-macrophages,[52] was negligible in our samples. Therefore, the toxicity observed was probably caused at least in part by 7β-OH-C and its precursors.

Relevance to Atherosclerosis

This direct comparison of LDL oxidation by different cell types *in vitro* cannot answer the outstanding question of their relative contribution

in vivo, which presumably depends on a variety of factors, including the cellular composition of the plaque. The fatty streak consists mainly of lipid-laden foam cells of monocytic origin,[54] suggesting that monocyte-macrophages may be mainly responsible for LDL oxidation at this stage of lesion development. Jonasson and colleagues estimated that advanced lesions contained about equal numbers of SMC and Mø (29% and 28%) and about 14% T-cells,[55] but routine histology shows widely varying proportions of SMC and Mø in different lesions. The contribution of each cell type to LDL oxidation would presumably depend on the cell composition of the individual plaque.

7β-OH-C and 26-OH-C have been found in lipid extracts of human lesions of all stages.[45] Fatty streaks contained the highest concentration of 7β-OH-C relative to cholesterol and the lowest ratio of 18 : 2 to 18 : 1, suggesting that this category of lesion experiences the greatest concentration of free radical-induced oxidation.[45] In normal diffusely-thickened adult intima, oxysterol levels were extremely low or undetectable,[45] arguing against any major involvement of SMC in oxidation in the absence of plaques. However, epitopes of oxidised LDL have been detected in SMC-rich diffuse intimal thickening,[56] and even minor degrees of SMC-mediated LDL oxidation at this stage might be significant, for example by contributing to monocyte recruitment.[5] 26-OH-C, a product of the enzyme cytochrome P-450 sterol 26-hydroxylase[46,47] rather than of free radicals, was found to be more abundant in advanced macrophage-rich lesions than in macrophage-poor (fibrous) lesions.[45] The failure to find 26-OH-C in our cultures may be due to lack of expression of this enzyme in our culture conditions.[57]

In the fatty streak, therefore, cell-mediated oxidation of LDL may be mainly due to the activity of monocyte-macrophages. Smooth muscle cells that have migrated into the intima during lesion progression may presumably also contribute. Endothelial cells, outnumbered by the other cell types at most stages of lesion development, probably contribute only marginally, but a role in the earliest stages of lesion development can not be excluded. Lymphocytes probably contribute only to a minor extent because of their relatively low oxidative capacity,[23] except perhaps in lesions in which they are numerous.[55]

The obvious difficulties in extrapolating from results in these cell cultures to the human lesion are further compounded by the enormously long time-scale of lesion development. In general, *in vitro* studies suggest that other possible confounding factors might include differences in pH and availability of oxygen, transition metals, antioxidants and oxidisable lipids in the different microenvironments of the lesion. These factors require stringent investigation.

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