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EDTA DIFFERENTIALLY AND INCOMPLETELY INHIBITS COMPONENTS OF PROLONGED CELL-MEDIATED OXIDATION OF LOW-DENSITY LIPOPROTEIN

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The extent to which cells can oxidize LDL may be underestimated because of the use of standard and arbitrary 24 hour *in vitro* incubations of cells with LDL. Such incubations have resulted in inconsistent results regarding the ability of cell-mediated LDL oxidation to generate relatively advanced oxidation products such as 7-ketocholesterol (7-KC). We studied prolonged oxidation of low density lipoprotein (LDL) by mouse peritoneal macrophages using HPLC measurement of cholesterol, cholesteryl esters and their oxidation products 7-KC and cholesteryl linoleate hydroperoxide (CL-OOH). Cell-mediated oxidation in Ham's F10 consistently followed the successive stages previously described during 24 hour-10 μM copper-mediated LDL oxidation, always generating 7-KC if allowed to proceed for sufficient time. The degree of inhibition of LDL oxidation achieved by metal chelators EDTA and DTPA at more advanced stages of cell-mediated LDL oxidation was not predictable from the published effects of such chelators upon early stages of metal-mediated and cell-mediated LDL oxidation. EDTA and DTPA only incompletely prevented the consumption of cholesteryl esters and the loss of preformed CL-OOH when added after cell-mediated LDL oxidation was established, while effectively concurrently inhibiting the generation of 7-KC. These data indicate that progressive cell-mediated peroxidation of LDL cholesteryl esters and decomposition of CL-OOH may be less dependent upon a continuing supply of redox active metals than is the generation of 7-KC. In addition, they confirm the plausibility of prolonged cell-mediated oxidation of LDL as a source of oxysterols found in human atherosclerotic plaque, and imply that active redox cycling of metals is particularly important for their generation *in vivo*.

KEY WORDS: EDTA, DTPA, macrophages, oxidation, low density lipoprotein, 7-ketocholesterol, cholesteryl ester hydroperoxide.

INTRODUCTION

Oxidation of low density lipoprotein (LDL) is likely to be important in the process of atherosclerosis.[1,2] Cell-mediated oxidation of LDL has been demonstrated in various media, including Ham's F10,[3,4] which supplies copper and iron, and metal-supplemented Dulbecco's Modified Eagles medium DME,[3,5,6] with endothelial cells, smooth muscle cells and macrophages.[1] The presence of transition metals has been found to be essential for cell-mediated-oxidation, and incubation of LDL in cell-free but copper-supplemented medium reproduces some of the changes characteristic of cell-mediated oxidation.[3] Consequently, *in vitro* studies of LDL oxidation have commonly used cell-free copper-mediated oxidation of LDL.[6] In addition, other systems, involving the

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exposure of LDL to defined oxygen-centred free radicals,[7] to peroxyl radical generators such as AMVN (2,2′-azobis-2,4-dimethylvaleronitrile) and AAPH (2,2′-azobis-amidinopropane hydrochloride),[8] to air[9] and to lipoxygenase[10] have been successfully used to generate oxidized LDL, although they may each produce a different peroxidation profile.

Hydroxylation products of arachidonic, linoleic and oleic acids have been demonstrated to arise during oxidation of LDL using GC, HPLC and MS techniques.[11,12] However, cholesterol oxidation products have required separate analyses using GC-MS or TLC.[13,14,15] None of the methods currently commonly used for the study of cell-mediated oxidation, such as the thiobarbituric acid reactive substances assay (TBARS),[16] iodometric assay,[17] and HPLC-chemiluminescence detection[18] provides the continuous assessment of defined products of LDL oxidation from early to late stages of oxidation. All the above assays focus upon the detection of early products, specifically lipid hydroperoxides. The most commonly used of these, the TBARS assay, is not specific for peroxides and is very insensitive compared with direct measures of lipid peroxide formation during LDL oxidation.[11,19,20] The conjugated diene assay does measure both early and later oxidation products, an initial maximum corresponding to the accumulation of lipid hydroperoxides, and a later maximum corresponding to unspecified unsaturated carbonyls.[21] However the specific identity of compounds measured at these times, and their generation under different conditions has been incompletely defined.

We have recently published a method for accurately defining the degree of copper-mediated LDL oxidation, which employed reversed phase high performance liquid chromatography (HPLC) with 234 nm and 210 nm absorbance detection.[22] 10 μM cupric chloride promoted the rapid oxidation of LDL. This oxidation could be described in four stages (involving the consumption of cholesteryl esters, the generation and subsequent decomposition of cholesteryl linoleate hydroperoxide (CL-OOH), and the generation of 7-ketocholesterol (7-KC)) and comparisons between samples at any time point could be simplified by deriving a numerical aggregate of these parameters (Lipoprotein Oxidation Index, L.O.I.). Within 24 hours a typical profile of advanced oxidation products of cholesterol and cholesteryl esters was generated, of which the most abundant detectable product was 7-KC.

While some studies have indicated that 7-KC is generated during cell-mediated LDL oxidation,[14] others have suggested it is exclusive to copper-mediated oxidation.[23] The mechanisms underlying the generation of such oxysterols are of importance as they have been identified in atherosclerotic plaque.[24] Further, while EDTA has been demonstrated to inhibit cell-mediated LDL oxidation over 24 hours (for example reference[3]) its effect upon lipid components has usually been determined by the use of indirect measures of lipid peroxidation, such as the TBARS assay. As far as we are aware, its effect upon the generation of individual lipid components of advanced cell-mediated LDL oxidation has never been studied.

These issues were approached by applying HPLC separation to study cell-mediated LDL oxidation. This was found to proceed slowly, but over incubations of longer duration followed stages similar to those described for 10 μM copper-mediated LDL oxidation, and ultimately also generated 7-KC and other advanced oxidation products. The metal-dependence of cholesteryl ester loss, CL-OOH generation and decomposition, and 7-KC generation during cell-mediated LDL peroxidation was examined. The metal chelators EDTA and DTPA substantially inhibited progression of lipid peroxidation even when added well after its onset. However, these agents differentially affected the individual products of mild and advanced LDL oxidation.

MATERIALS AND METHODS

LDL Isolation

LDL (1.05 > d > 1.02 g/ml) was isolated from plasma of normolipidaemic, healthy subjects using two sequential centrifugation steps at 10° C using a Beckman L8-M centrifuge and a VTi 50 rotor, at 50 000 R.P.M. (242 000 g) for 2.5 hours, then a Ti 70 rotor, at 50 000 RPM, for 22 hours.[25] LDL was always isolated from individual donors, not from pooled plasma, and multiple incubations of a single preparation of LDL were used in each experiment. The LDL was dialysed against 4 × 1 L deoxygenated phosphate-buffered saline (PBS, calcium and magnesium free; Flow laboratories) containing 0.1 mg/ml chloramphenicol (Boehringer Mannheim) and 1.0 mg/ml (2.7 mM) ethylenediaminetetraacetic acid (EDTA; British Drug Houses). The LDL was stored in the dark at 4° C under nitrogen until use (within 7 days).

Prior to oxidation, LDL was dialysed against 3 × 1L PBS (LDL:PBS, 1:200, v:v) containing chloramphenicol (0.1g/l) and Chelex-100 (1g/l), then 1 × 1L PBS containing chloramphenicol only, over a total of 16 hours to remove EDTA.

Cell Culture

Resident macrophages were isolated from 6 week-old QS mice, after asphyxiation using carbon dioxide gas, by peritoneal lavage with ice-cold Dulbecco's Minimum Essential Medium (DMEM; Gibco Cat 320-1885 AJ) containing 0.38% (w/v) sodium citrate, penicillin G (50 Units/ml) and streptomycin (50 μg/ml). Equal numbers of male and female mice were used, and mice weighed between 20–25g. The isolated cells were immediately plated in 24 mm-diameter tissue culture wells (Costar) at 3.0×10^6 cells per well, incubated at 37° C for 1–2hrs, then washed four times with pre-warmed PBS to remove non-adherent cells.

LDL Oxidation

After washing, cells were incubated in 1 ml of Ham's F10 medium (Gibco) containing 50 μg LDL protein, without added antibiotics, for up to 48 hours. To ensure cell viability for experiments extending beyond 48 hours, fresh macrophages were harvested and plated at 48 hours, and LDL in Ham's F10 medium was transferred from the initial cell-culture dishes to the dishes containing fresh macrophages and then incubated for up to a further 48 hours. As a control, LDL samples were concurrently incubated in Ham's F10 medium without cells for the same duration. LDL samples were removed from these replicate cell culture dishes at specified times during incubation.

In experiments in which metal chelators EDTA or diethylenetriaminepentaacetic acid (DTPA, Sigma) were added to LDL incubations, the chelators were added to cell culture dishes containing Ham's F10 and LDL (with or without cells) at a final concentration of 250 μM. The additions were made at times specified in Results.

Lipid Extraction

0.8 ml of LDL (in Ham's F10 medium) was removed from the culture dish and spun in an Eppendorf centrifuge at 4° C for 2 minutes at 14 000 rpm (16 000 g) to remove any detached cells. 0.4ml of the supernatant was mixed with 0.6ml ice-cold PBS in the presence of 20 μM butylated hydroxytoluene (BHT; British Drug Houses) and 2 mM

EDTA, and was extracted into methanol (2.5 ml) then hexane (10 ml).[22] Samples were stored after extraction at −80° C until analysis which was usually performed within 7 days.

As previously published,[22] cholesterol, cholesteryl ester, 7-KC, and cholesteryl linoleate hydroperoxide (CL-OOH) standards were confirmed to be extracted with approximately 100% efficiency.

HPLC Analysis

The method of analysis of cholesterol, cholesteryl esters and their oxidation products has recently been published in detail.[22] In brief, unoxidized cholesterol and cholesteryl esters were separated using reversed phase high performance liquid chromatography (HPLC) at room temperature on a C-18 column (Supelco), using an eluent of acetonitrile/isopropanol (30/70, v/v) and detected by their 210nm absorbance using an Activon UV 200 absorbance detector. Oxidation products, in particular CL-OOH and 7-KC, were also analysed using HPLC on a C-18 column, but were separated with a solvent system of acetonitrile/isopropanol/ water (44/ 54/2, v/v/v) and detected by their absorbance at 234nm. Results are expressed as nmol per mg of LDL protein and are presented as mean ± standard deviation (S.D.) of three individual incubations.

The results of HPLC analyses of cholesterol and cholesteryl esters, 7-KC and CL-OOH were combined to give a single numerical measure of the degree of lipoprotein oxidation.[22] This lipoprotein oxidation index, or L.O.I., is calculated using the ratio

$$(24 \times \text{7-KC} + \text{CL-OOH})/(\text{C} + \text{CE})$$

where 7-KC (7-ketocholesterol), CL-OOH (cholesteryl linoleate hydroperoxide), C (unesterified cholesterol), and CE (the sum of the measured cholesteryl esters - cholesteryl arachidonate, cholesteryl linoleate, cholesteryl oleate and cholesteryl palmitate) are all in molar units. Individual calculations were made for each cell culture dish from which LDL was removed, and results are expressed as the mean ± S.D. of three incubations.

Statistical Analysis

Where indicated, statistical comparisons were made using Student's t-test for unpaired data. A statistically significant difference was inferred if $p < 0.05$.

Protein Estimation

The protein content of LDL samples was measured using the bicinchoninic acid BCA method (Sigma) using bovine serum albumin (BSA) as standard. Standards were prepared in water, samples were incubated for 60 minutes at 60° C and absorbance was measured at 562 nm.

RESULTS

(A) Analysis of Cell-Mediated and Cell-Free LDL Oxidation

LDL which had not been exposed to copper or cells did not contain CL-OOH detectable at 234 nm (Figure 1(a),(b) – 0 hours; limit of sensitivity approximately 5 ng,

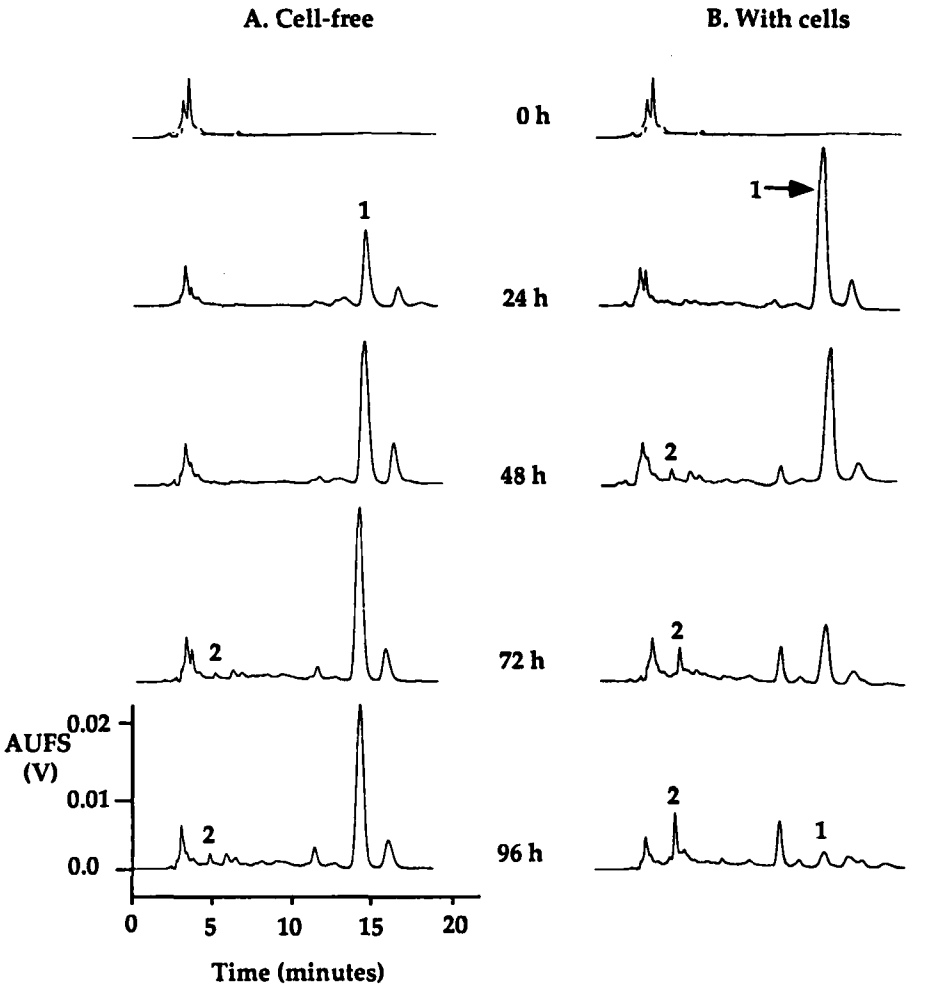

FIGURE 1 HPLC separation of oxidation products generated during 96 hour incubation of LDL, with or without macrophages, in Ham's F10 medium.

Fresh human LDL was simultaneously incubated in Ham's F10 medium without (A) or with (B) mouse peritoneal macrophages for up to 96 hours, as described in Methods. Samples of LDL in Ham's F10 were extracted (at the times specified) and analysed for 234 nm absorbance after reversed phase HPLC separation using a solvent of acetonitrile/ isopropanol/ water (44/54/2; v/v/v).

Key: Peak "1" is cholesteryl linoleate hydroperoxide and peak "2" is 7-ketocholesterol. AUFS (V), area units full scale (volts).

equivalent to 1.2 nmole CL-OOH/mg LDL protein or 0.7 molecules CL-OOH per molecule of LDL). Within 24 hours incubation in Ham's F10 alone (Figure 1(a)), LDL contained 62.3 ± 4.4 nmol/mg of CL-OOH; while in LDL incubated in Ham's F10 with cells for the same period contained 170.9 ± 6.5 nmol/mg of CL-OOH (Figure 1(b)). Over 96 hours, LDL incubated in Ham's F10 alone underwent increasing oxidation with a rise in the content of CL-OOH, and a generation of small quantities of 7-KC and other oxidation products. Others have shown that Ham's F10[3,5,28] and Ham's F12[26] media are capable of causing substantial cell-free oxidation, especially when LDL

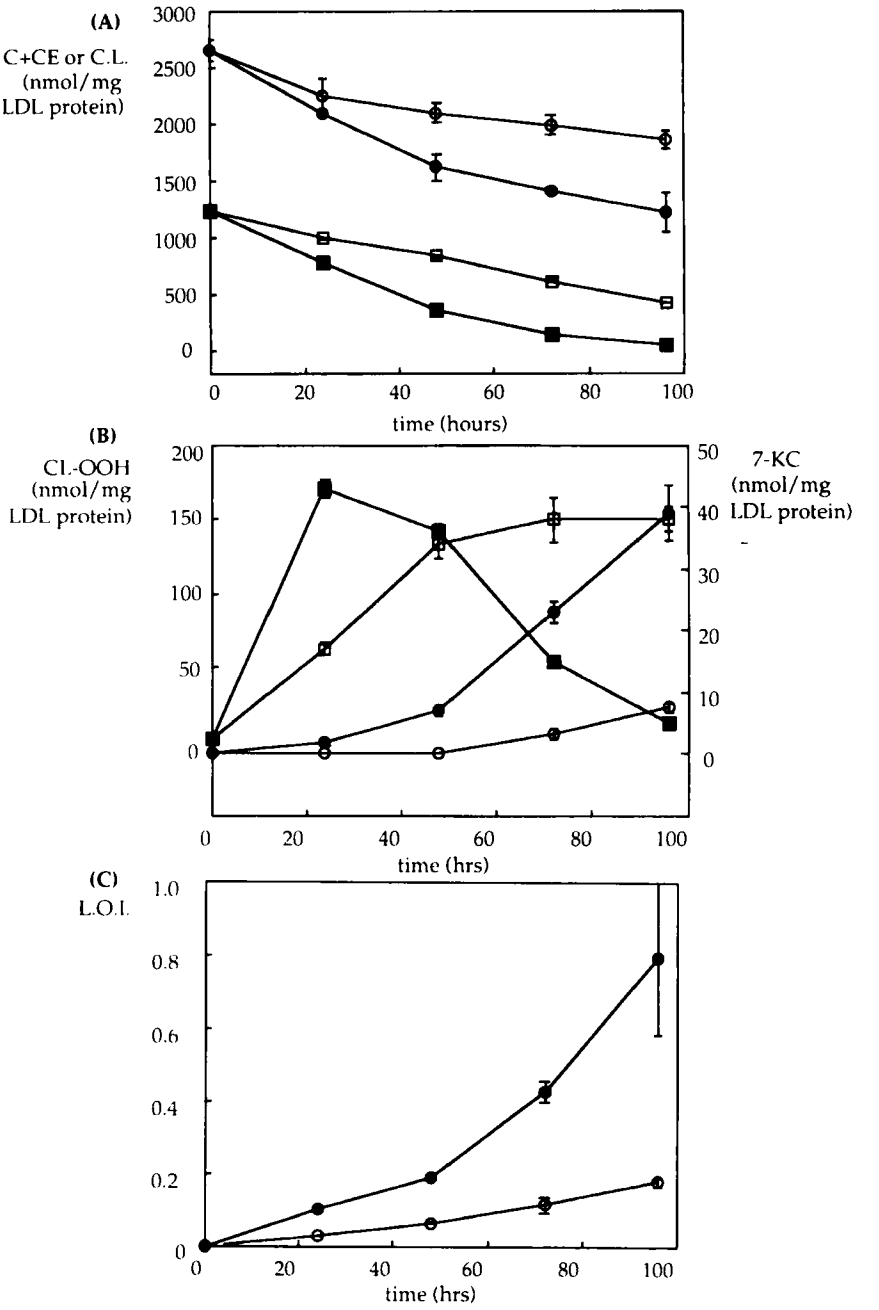

FIGURE 2 Changes in cholesterol, cholesteryl ester, cholesteryl linoleate hydroperoxide and 7-ketocholesterol content of LDL during incubation in Ham's F10 medium.

(A) Fresh human LDL incubated in Ham's F10 medium for up to 96 hours with (-●-) or without (-○-) mouse peritoneal macrophages, was analysed for total cholesterol and cholesteryl esters (including cholesteryl linoleate; C+CE) by HPLC as described in Methods. The quantity of cholesteryl linoleate (C.L.) in LDL incubated with (-■-) or without (-□-) mouse peritoneal macrophages is also indicated separately.

(B) LDL as in (A), analysed for cholesteryl linoleate hydroperoxide (CL-OOH; -■- with cells, -□- without cells) and 7-ketocholesterol (7-KC; -●- with cells, -○- without cells) by HPLC as described in Methods.

(C) LDL incubated in Ham's F10 medium for up to 96 hours, with (-●-) or without (-○-) mouse peritoneal macrophages, analysed for Lipoprotein Oxidation Index (LOI) which was derived from data shown in (A) and (B), as described in Methods.

Each point represents the mean ± S.D. of triplicate incubations of a single preparation of LDL.

protein concentrations were lowered (20 μg/ml) or if incubations were extended to 48 hours.[3] Thus, our finding that LDL was oxidized in Ham's F10 at 50 μg LDL protein/ml (a relatively low concentration) is consistent with published data.

In agreement with previous studies, cell-mediated LDL oxidation was much more rapid than oxidation of LDL in medium without cells. LDL incubated in F10 alone for 96 hours displayed a similar chromatographic profile to that seen in the LDL incubated with F10 and cells for only 48 hours (Figures 1 and 2). The content of CL-OOH in LDL incubated in F10 with cells reached a maximum level at or before 24 hours, such that only 13.0 ± 2.5 nmol of CL-OOH remained per mg of LDL protein by 96 hours. By this stage, a large quantity of 7-KC (39.0 ± 4.6 nmol/mg LDL protein) had been generated, together with a number of other as yet incompletely characterised oxidation products of cholesterol and cholesteryl esters. Incubation of LDL for only 24 hours (as is normally the case in studies of cell-mediated oxidation of LDL) would have precluded seeing the sequence of CL-OOH increase and decrease and 7-KC generation with progressive cell-mediated LDL oxidation, which is identical to that described for copper-mediated LDL oxidation.[22] The corresponding loss of cholesteryl esters at these times confirms that the generation of 7-KC and the loss of cholesteryl linoleate hydroperoxide in the cell-incubated LDL reflected increasingly extensive oxidation. As expected, the most readily oxidized cholesteryl esters were cholesteryl linoleate and cholesteryl arachidonate, while unesterified cholesterol, cholesteryl oleate and cholesteryl palmitate were much less affected during this period (data not shown).

With very mild oxidation, CL-OOH content best distinguished more oxidized cell-incubated LDL from cell-free incubations of LDL, but as incubations continued, less-extensively oxidized cell-free incubations of LDL contained more peroxide than cell-incubated LDL. In these cases 7-KC content was a superior index of advanced oxidation and was consistently greater after cell-mediated oxidation than cell-free incubations of LDL at all times in these experiments, although it was not sufficiently detectable after mild oxidation to be useful at early stages. The loss of cholesteryl ester, rise and fall of CL-OOH, and the generation of 7-KC were expressed as a single numerical index, L.O.I., which indicated that LDL incubated in F10 alone was not as extensively oxidized as that incubated with cells at every time point.

To ensure that such stages of LDL oxidation could be achieved in LDL derived from a number of donors, we studied the simultaneous cell-mediated oxidation of LDL from three individual donors (Figure 3). Using a single preparation of Ham's F10 medium, the three LDL preparations all followed the same stages of CL-OOH generation, cholesteryl ester consumption and 7-KC generation described above. The rates of oxidation of all three LDL preparations were generally faster than that shown in Figure 2, and there was also slight variation between the LDL preparations in Figure 3. Over many experiments, slight variations in extent of oxidation were observed between different preparations of LDL, consistent with previous literature regarding different rates of oxidation of LDL from different donors.[6,27,28] Some variation in the metal content of individual preparations of commercially supplied Ham's F10 medium could also contribute to interexperiment variation. Consequently, a single preparation of Ham's F10 medium was used in each individual experiment for comparisons of LDL oxidation with and without macrophages.

(B) Effect of EDTA Upon Cell-Mediated and Cell-Free LDL Oxidation.

The addition of EDTA or histidine during the propagation of copper-mediated LDL oxidation prevented further increases in TBARS over a total of 3 hours.[29] EDTA (50

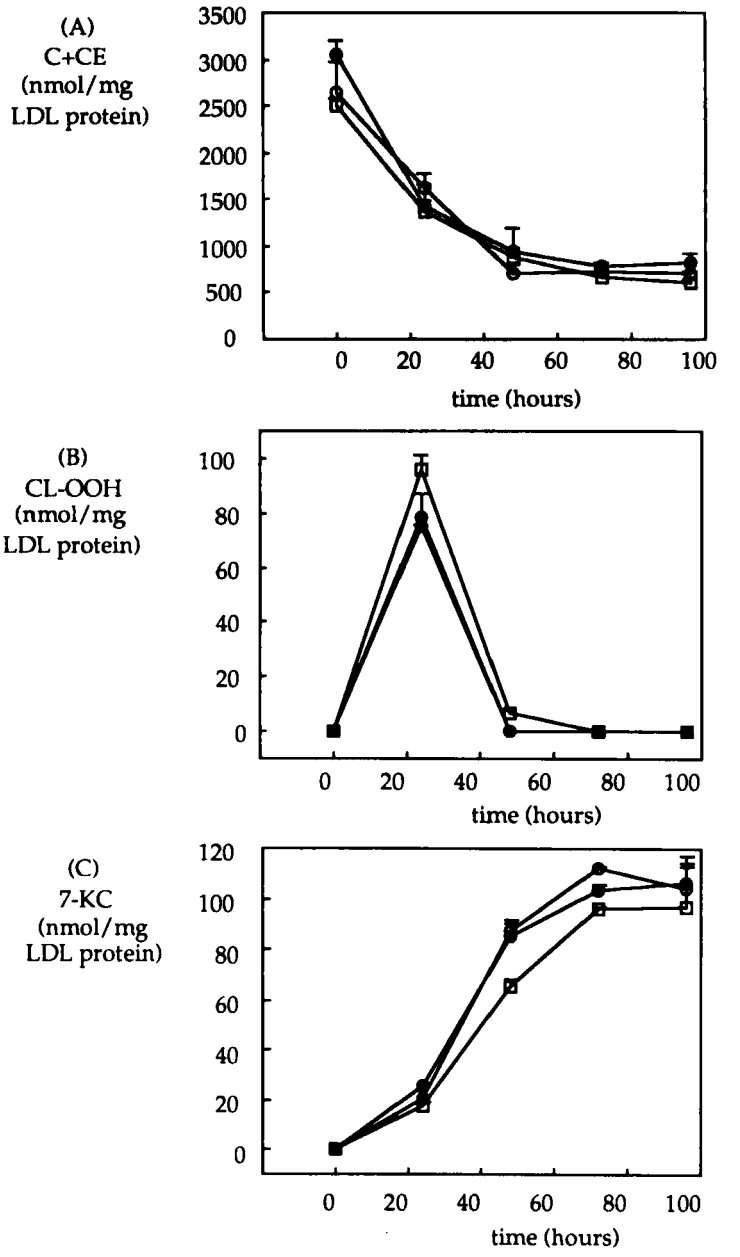

FIGURE 3 Comparative oxidation of LDL from individual donors.

LDLs from three individual donors were incubated in Ham's F10 medium for up to 96 hours with mouse peritoneal macrophages. A single batch of Ham's F10 and a single cell harvest was used for all three LDL preparations. LDL was analysed for total cholesterol and cholesteryl esters (including cholesteryl linoleate; C+CE, panel (A)), cholesteryl linoleate hydroperoxide (CL-OOH; panel (B)), and 7-ketocholesterol (7-KC; -panel (C)).

Each point represents the mean ± S.D. of triplicate incubations of each LDL preparation, and each preparation is represented by the same symbol in all three panels.

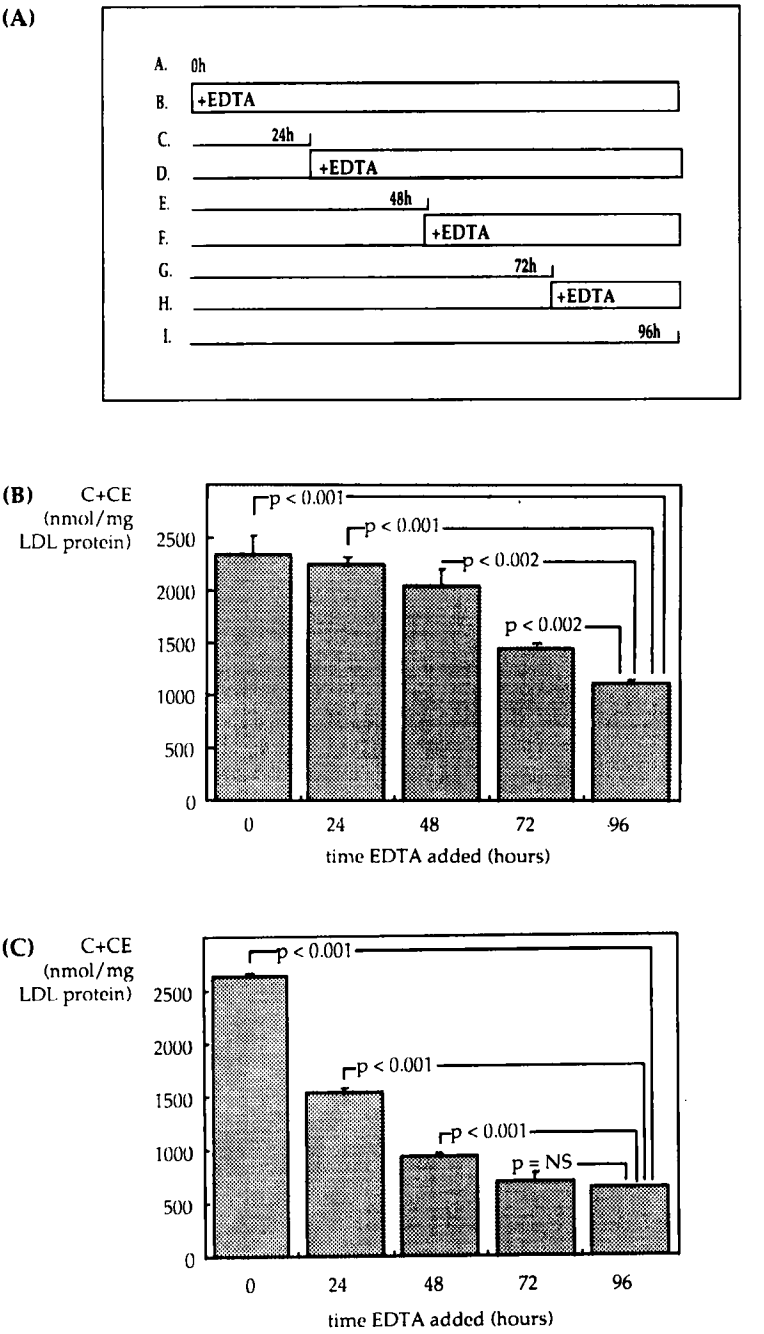

FIGURE 4 EDTA prevents the consumption of cholesterol and cholesteryl esters during incubation of LDL in Ham's F10 medium.

(A) Schema for experiments described in Figures 4 (b) (c) and Figures 5 and 6. EDTA was added to incubations of LDL in Ham's F10 after 0, 24, 48, or 72 hours (samples B, D, F, H respectively) and incubations were continued for a total of 96 hours. Samples were removed from control incubations at the time of EDTA addition (samples A, C, E, G). Sample I corresponds to incubations lasting 96 hours to which EDTA was not added. The 0 h point of EDTA addition and sample extraction is actually 15 minutes after the addition of LDL to Ham's F10.

(B) and (C) LDL (50 μg/ml) was incubated in Ham's F10 for 96 hours, without (B), or with (C), mouse peritoneal macrophages. EDTA was added at the specified times to cell-culture dishes at a final concentration of 250 μM, and all incubations shown were continued until 96 hours. Statistical annotations (unpaired Student's T-test) compare total LDL cholesterol and cholesteryl esters (C+CE) after 96 hours without EDTA to C+CE at 96 hours after EDTA had been added at 0, 24, 48 or 72 hours. Results represent the mean ± S.D. of triplicate incubations of a single preparation of LDL.

μM) inhibited cell-mediated oxidation in Ham's F10 over 24 hours (as assessed by TBARS assay and LDL protein modification) when EDTA was added at the onset of incubations.[3] However, the inhibition by EDTA of later stages of metal-mediated or cell-mediated LDL oxidation and the generation of advanced oxidation products such as 7-KC were not studied in either of these studies. We investigated the effect of EDTA on LDL incubated in Ham's F10 (with or without cells) for 0, 24, 48, 72 or 96 hours. At each time some LDL samples were removed, while to other samples EDTA was added and the incubation was continued until 96 hours (summarised in Figure 4(a)).

Cholesterol and Cholesteryl Esters

LDL samples to which EDTA had been added during the incubation contained more cholesterol and cholesteryl esters than 96 hour incubations to which EDTA had not been added (Figure 4 (b) and (c)). As expected, the preservation was greater with earlier EDTA additions. As the oxidation in the presence of cells was more vigorous than without cells, as discussed above, the quantity of preserved cholesterol and cholesteryl ester was less at each time in the presence of cells. These data suggest that the availability of redox-active metal influences the extent of total cholesterol and cholesteryl ester oxidation achieved by cells in Ham's F10 and by Ham's F10 alone even after the oxidation process is well-advanced.

Cholesteryl Linoleate Hydroperoxide

Because the depletion of total cholesterol and cholesteryl ester is a relatively crude measure of lipid oxidation, we analysed in detail the levels of three components of the LDL lipid peroxidation process (Figure 5). CL-OOH is a sensitive marker of mild oxidation, and, lipid hydroperoxide decomposition may be central to the modification of LDL protein and subsequent cellular uptake of oxidized LDL.[28,30]

As observed in previous experiments (Figures 1-3), CL-OOH accumulation was followed by a decline during the 96 hour incubation, and both processes evolved more rapidly with cells (Figure 5(b)) than without (Figure 5(a)). EDTA substantially inhibited the decline of preformed CL-OOH compared with matching EDTA-free incubations. However, the preservation was incomplete in cell-free incubations, and even less so in incubations with cells. For example, LDL incubated with cells to which EDTA had been added at 24 hours contained 168 ± 7.6 nmol CL-OOH per mg LDL protein at the time of EDTA addition, but only 69.7 ± 7.3 nmol/mg after a further 72 hours (a drop of of 59% between 24 and 96 hours).

The inefficient protection of CL-OOH by EDTA in both cell-free and cell-containing incubations could be due to incomplete chelation of redox active iron and copper by EDTA because of binding of metals by phosphate and histidine in F10 medium. However, there was an 80:1 molar excess of EDTA to iron and 2×10^4:1 molar excess of EDTA to copper (according to manufacturers specifications of the content of these metals in Ham's F10) and EDTA-iron and EDTA-copper complexes are extremely stable.[31] While contaminating copper could be greater than the specified 10 nM, samples of Ham's F10 used in these experiments were confirmed to contain less than 50 nM by Inductively Coupled Plasma Atomic Emission Spectrometry (Mr. R. Finlayson, Dept. Analytical Chemistry, University of NSW), thus the molar ratio of EDTA to copper in these experiments would be expected to be at least 5×10^3:1.

Decline in CL-OOH in the presence of EDTA was most evident in the presence of cells. Cells may be capable of consuming peroxides in a metal-independent manner not

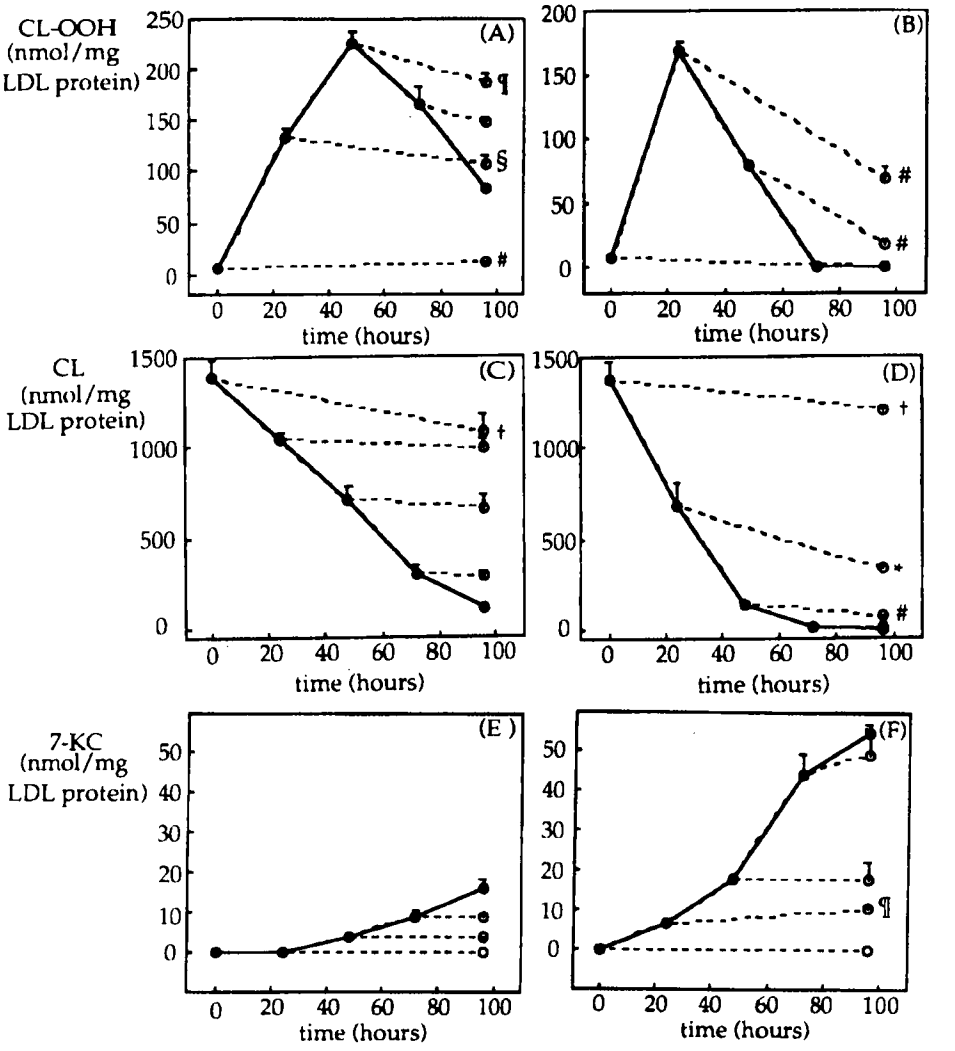

FIGURE 5 EDTA incompletely and differentially inhibits components of prolonged cell-mediated LDL oxidation.

LDL (50 μg/ml) was incubated in Ham's F10 for 96 hours, without (panels (A), (C) and (E)), or with (panels (B), (D), and (F)), mouse peritoneal macrophages. EDTA (dashed line, open symbol) was added at 0, 24, 48, or 72 hours to some cell-culture dishes at a final concentration of 250 μM, and all incubations (with or without EDTA) were continued until 96 hours (see Figure 4(a)). Incubations not receiving EDTA are represented by the solid line and solid symbol. Statistical annotations compare the cholesteryl linoleate hydroperoxide (CL-OOH; panels (A) and (B)), cholesteryl linoleate (CL; panels (C) and (D)), and 7-ketocholesterol (7-KC; panels (E) and (F)) content of samples extracted at 96 hours to which EDTA had been added at 0, 24, 48, or 72 hours, with that of samples extracted when EDTA was added. Thus in panel (A), the dashed line beginning at 24 hours (marked "§" at 96 hours), represents the change in CL-OOH content from 24 hours to 96 hours in the presence of EDTA, and "§" implies that the two values connected by the dashed line are significantly different. Results represent the mean ± S.D. of triplicate incubations of a single LDL preparation, and where error bars are not visible they are enclosed by the symbol. Values marked "*", "§" "¶" and "#", indicate $p < 0.05$, 0.02, 0.01 and 0.001 respectively for comparison of 96 hour EDTA value with value at the time of EDTA addition. The decrease in CL values marked "†" in panels (C) and (D) were not reproduced in later experiments (discussed in text).

related to oxidation. For example, we have observed clearance of LDL CL-OOH by mouse peritoneal macrophages in a medium which did not permit LDL oxidation (DMEM and 10% LPDS, Kritharides et al., unpublished observations), and lipid hydroperoxide clearance by Hep G2 cells has been previously described.[32]

In no sample where EDTA was added at 24 hours or later did the level of CL-OOH show any net increase, even though in the case of cell-free incubations without EDTA the peroxide content of the LDL was still rising (Figure 5(a)). While an increase of CL-OOH in the interval between EDTA addition and extraction at 96 hours may have been missed, it is also possible that EDTA was much more effective at stopping the net generation of CL-OOH than preventing its consumption. The lack of any increment in CL-OOH may explain the apparently "complete inhibition" of lipid peroxidation observed following the addition of EDTA using different, indirect peroxide-detecting systems described in previous literature.

Cholesteryl Linoleate Consumption

Cholesteryl linoleate is a major substrate for oxidation during LDL incubation in Ham's F10, with and without cells (Figure 2). EDTA, in general, effectively inhibited the loss of cholesteryl linoleate during cell-free incubations. In particular, the addition of EDTA at 24 and 48 hours in cell-free incubations prevented the subsequent loss of cholesteryl linoleate (Figure 5(c)), during which time there was a minor but significant loss of CL-OOH (Figure 5(a)).

EDTA incompletely inhibited the consumption of cholesteryl linoleate during incubation of LDL with cells. For example (Figure 5(d)), LDL incubated with cells to which EDTA was added after 24 hours contained 679 ± 128 nmol cholesteryl linoleate/mg LDL protein at 24 hours, and 335 ± 16 nmol/mg by 96 hours (a decrease of 51%). In comparison, cell-free incubations to which EDTA had been added at 24 hours had lost only 5.5% of cholesteryl linoleate present by 96 hours (1041 ± 39 nmol/mg at 24 hours, and 991 ± 45 nmol/mg at 96 hours).

Ongoing loss of cholesteryl linoleate was most clear when EDTA was added during the period of most rapid cholesteryl linoleate consumption (e.g. EDTA added at 24 hours, at which time the average rate of cholesteryl linoleate consumption for the preceding 24 hours had been 29 nmol of cholesteryl linoleate per mg LDL protein per hr). This suggests that progressive oxidation at 24 hours in the presence of cells and EDTA may be due to lipid peroxyl and alkoxyl radicals generated prior to the addition of EDTA. Such radicals could cause lipid peroxidation chain reactions[20,33,34] without a continuing requirement for metals.

The loss of cholesteryl esters and CL-OOH could not be attributed to LDL uptake via cellular LDL or "scavenger" receptors. Less than 10% of acetylated (AcLDL) and extensively-oxidized LDL (OxLDL) supplied in medium at a concentration of 10 μg/ml was taken up by mouse peritoneal macrophages in 24 hours.[35] As the total uptake of native LDL by mouse peritoneal macrophages is much less rapid than that of either OxLDL or AcLDL,[36,37] less than 5% of the total native LDL in the culture medium could be actually endocytosed over 24 hours. Even if it is assumed that all LDL present after 24 hours incubation with cells is a suitable ligand for the scavenger receptor (almost certainly an overestimate), then not more than 10% decline in cholesteryl esters every 24 hours could be accounted for by endocytosis (total loss between 24 and 96 hours of 30%) which is still much less than the 50% decrease in cholesteryl linoleate observed over this time. Moreover, we analysed cell extracts after these incubations and found no evidence of cellular accumulation of cholesteryl esters (data not shown).

That the losses in the presence of cells were attributable to oxidation and not endocytosis of oxidized LDL is most clearly demonstrated by the differences in loss of unesterified cholesterol and individual cholesteryl esters. Between the addition of EDTA at 24 hours and extraction at 96 hours, cholesteryl linoleate decreased by 51%, cholesteryl oleate decreased by 27%, cholesteryl palmitate decreased by 16% and free cholesterol did not decrease (808 ± 5 nmol cholesterol/mg LDL protein at 24 hours, and 811 ± 18 nmol cholesterol/mg LDL protein at 96 hours). If LDL endocytosis were responsible for the loss of cholesteryl linoleate, equal losses of cholesterol and all cholesteryl esters should have been observed during this period.

An isolated unexplained loss of cholesteryl linoleate when EDTA was added at the onset of incubation (Figures 5(c) and (d), points marked "†") was not seen in repeated subsequent experiments (for example in Figures 6 (c) and (d), described below). While this could reflect oxidative loss, it is more likely to be an artefact of experimental procedure and will not be considered further.

7-Ketocholesterol

EDTA very effectively inhibited the generation of 7-KC, in both cell-free (Figure 5(e)) and cell-containing incubations (Figure 5(f)), with the exception of a minor increase in 7-KC (in cell-containing incubations) following the addition of EDTA at 24 hours (Figure 5(f)). By 96 hours, LDL incubations to which EDTA was added at 0, 24, and 48 hours, contained significantly less 7-KC than samples to which EDTA had not been added ($p < 0.001$). The loss of CL-OOH and cholesteryl linoleate which had occurred with EDTA were much more marked than was any concurrent generation of 7-KC. Thus the generation of 7-KC appeared to be critically dependent upon the availability of redox-active metal at all stages of cell-mediated and cell-free LDL oxidation.

(C) Comparison of EDTA with DTPA

A previous study indicated that DTPA effectively inhibited smooth muscle cell-mediated LDL oxidation in iron supplemented Ham's F10 medium while EDTA promoted such oxidation at concentrations up to 100 μM (EDTA to iron ratio of up to 10:1).[38] It was unlikely that a pro-oxidant effect of EDTA was relevant in our studies since this was not observed when EDTA was added at 0h. Nevertheless, to exclude a pro-oxidant effect of EDTA as the cause for the continued loss of CL-OOH and loss of cholesteryl linoleate in its presence we compared EDTA and DTPA (both at 250 μM) upon cell-mediated and cell-free LDL oxidation (Figure 6). (The use of 1mM EDTA was attempted but was abandoned because of obvious and severe cell toxicity.)

Both EDTA and DTPA incompletely prevented the net loss of CL-OOH and the consumption of cholesteryl linoleate. There was very mild oxidation when the chelators were added at the start of incubation (with EDTA, for example, there was increase from 0 to 3.2 nmol CL-OOH/mg LDL protein in 96 hours with cells and from 0 to 16.9 nmol CL-OOH/mg LDL without cells, but without significant decrease in cholesteryl linoleate). In contrast, there were clear net losses of CL-OOH and cholesteryl linoleate when chelators were added at later times. Between 24 and 96 hours there was 74% net loss of CL-OOH in cell-containing incubations in the presence of EDTA and 65% net loss in matched incubations in the presence of DTPA (Figure 6(b)). Simultaneously, there was 40% loss of cholesteryl linoleate with EDTA and 32% loss in the presence of DTPA (insert Figure 6(d)). In the presence of chelators substantial losses of cholesteryl linoleate only occurred in cell-containing incubations. There was no increase in the

7-KC content of LDL in the presence of either chelator at any time (data not shown).

Several additional aspects of these data warrant brief mention. Firstly, the rate of LDL oxidation, with or without cells, was faster in Figure 6 than in Figure 5. This may explain the small loss of cholesteryl linoleate in cell-free incubations with EDTA between 24 and 96 hours (Figure 6(c)) if such loss is oxidative and is affected by the concentration of pre-formed lipid radicals at the time of addition of chelator. Secondly, the concentration of CL-OOH at 96 hours in cell-free incubations was consistently greater than that in cell-containing incubations when chelators were added at the start of incubation. This could be due to cellular clearance of peroxide or cellular promotion of oxidative decomposition of CL-OOH. Thirdly, while EDTA and DTPA exerted very similar effects in these experiments, the loss of CL-OOH and of cholesteryl linoleate were usually (but not always) slightly greater with EDTA than with DTPA. For example, in cell-free incubations in Figure 6(a), the CL-OOH content had decreased from 219 ± 4 nmol CLOOH/mg LDL protein at 24 hours to 147 ± 3 nmol CLOOH/mg LDL at 96 hours with EDTA and 168 ± 1 nmol at CLOOH/mg LDL 96 hours with DTPA ($p < 0.01$, DTPA vs EDTA). In general, however, any differences between the chelators were minor and not significant.

DISCUSSION

Two issues have been addressed in these experiments: (a) whether, cell-mediated LDL oxidation is capable of reliably generating advanced products of LDL oxidation, such as have been identified in human atherosclerotic plaque; and (b) whether the consumption of cholesteryl esters, the generation and consumption of CL-OOH and the generation of 7KC during LDL oxidation are equally prevented at all times by the addition of EDTA. 7-KC and other oxysterols such as 7-hydroxycholesterol have been described after metal-mediated oxidation of LDL,[13,14,15,22,23] and in atheroma from human[39,40] and rabbit[24] atherosclerotic plaque, but have been inconsistently described after *in vitro* cell-mediated oxidation of LDL.

The difference between LDL oxidation in Ham's F10 and cell-mediated LDL oxidation in Ham's F10 as described in this paper, and cell-free 10 μM copper-mediated oxidation of LDL previously described,[22] appears to be only one of degree. Under all three conditions, the oxidation of LDL follows the pattern of peroxide accumulation (specifically CL-OOH), followed by net peroxide loss and the generation of oxysterols (specifically 7KC). Incubation of LDL with cells facilitated the oxidation of LDL, and, in so doing, accelerated the production of 7-KC. Rather than a true qualitative difference, it is likely the conflicting previous literature is explained by different rates of LDL oxidation in various cell-culture systems. This conclusion is supported by a recent observation that bovine aortic endothelial cells inconsistently modified LDL by 24 hours, but consistently did so after 48 hours incubation with LDL.[41] Unpublished data from our laboratory (B. Garner, R. T. Dean, W. Jessup) has demonstrated that LDL incubated with 6 day old human monocyte-derived macrophages contains 7-KC within 8 hours, consistent with previous data,[14] and by 24 hours reaches the same stage of oxidation as that seen with mouse peritoneal macrophages after 96 hours.

That cell-mediated LDL oxidation under the low metal concentrations present in Ham's F10 medium (3μM iron and 0.01μM copper[5,6]), can reliably generate large quantities of 7-KC is of potential pathophysiological significance. Oxysterols identified in human plasma, including 7-KC, may be ingested from the diet.[42] However, in a recent study of cholesterol-fed rabbits,[24] 7-KC and 25-OH cholesterol accumulated in aortic

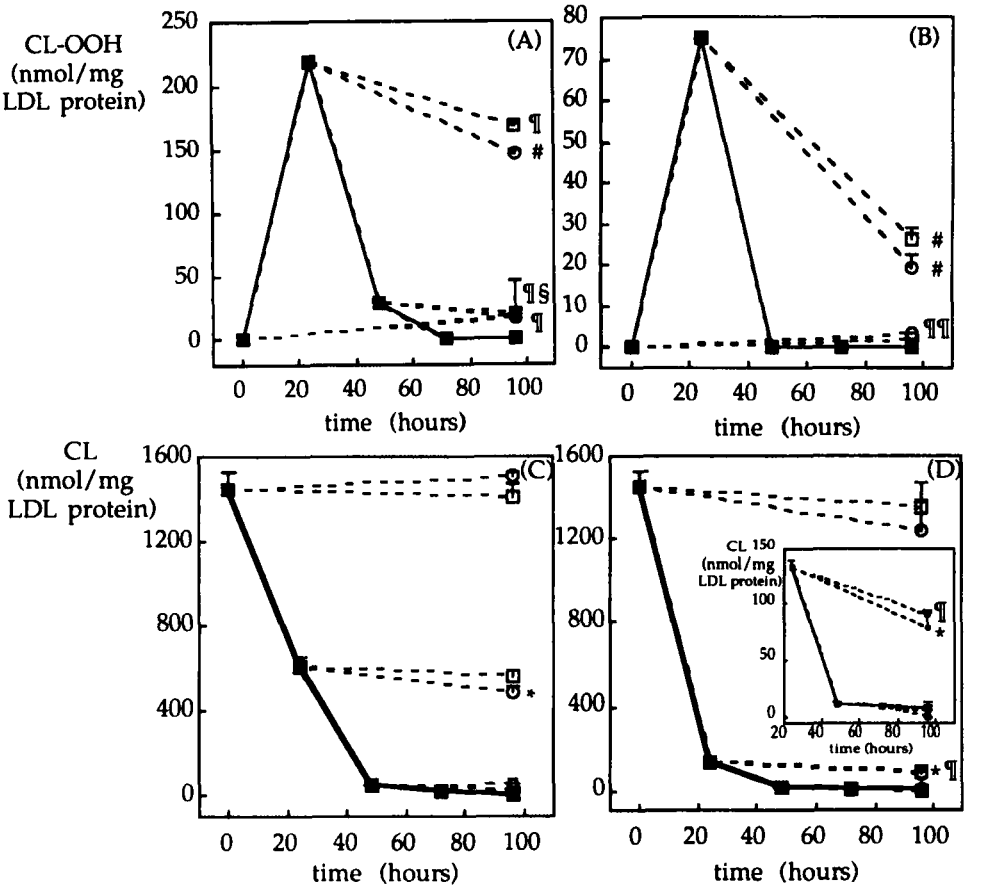

FIGURE 6 Comparative efficacy of EDTA and DTPA in inhibiting advanced cell-mediated LDL oxidation. The basic experimental design is the same as that in Figures 4 and 5. LDL (50 μg/ml) was incubated in Ham's F10 for 96 hours, without (panels (A), (C)), or with (panels (B), (D)), mouse peritoneal macrophages. EDTA (dashed line with open circle) or DTPA (dashed line with open square) were added at 0, 24, 48, or 72 hours to some cell-culture dishes at final concentrations of 250 μM, and all incubations (with or without chelators) were continued until 96 hours. Incubations not receiving EDTA or DTPA are represented by the solid line and solid symbol. Results represent the mean ± S.D. of triplicate incubations of a single LDL preparation, and where error bars are not visible they are enclosed by the symbol. Cholesteryl linoleate hydroperoxide (CL-OOH; panels (A) and (B)), and cholesteryl linoleate (CL; panels (C) and (D)) values marked "*", "§", "¶" and "#", indicate $p < 0.05$, 0.02, 0.01 and 0.001 respectively for comparison of either 96 hour EDTA or DTPA value with value at the time of chelator addition. Where the symbols and lines of samples receiving EDTA and DTPA overlap, a single significance symbol or the first of two significance symbols refers to samples receiving EDTA. The insert in panel (D) shows in greater detail the change in CL content with and without chelators between 24 and 96 hours with cells and is obtained unmodified from the data represented in panel (D).

tissue without being detected in cholesterol-containing feed, nor in plasma, suggesting they may have been generated in the arterial wall. Our data indicate that a process of slow cell-mediated LDL oxidation can explain the presence of 7-KC in human atherosclerotic plaque.

Our results indicate that the availability of redox-active metals significantly affects

the extent of cell-mediated oxidation even if oxidation is very advanced at the time of their chelation. Although this suggests that the propagation and decomposition phases of cell-mediated LDL oxidation may be dictated by metals present, this does not necessarily imply that the effect of metal chelation on cell-mediated LDL oxidation can be predicted by its effect in cell-free systems.

EDTA did not completely arrest peroxide decomposition or cholesteryl ester consumption during cell-mediated- or, to a lesser extent, cell-free-, LDL oxidation. EDTA can interact with iron to promote iron mediated microsomal peroxidation[43] and LDL oxidation.[44] However EDTA to iron ratios of 1:1 are optimal for oxidation[43] and EDTA to iron ratios greater than 2:1 inhibited cell-mediated LDL-oxidation in Ham's F10.[44] Heinecke et al. demonstrated enhanced LDL oxidation with EDTA to iron ratios of 1:1 or 10:1 (studies performed in Ham's F10 supplemented with 10 μM iron), but EDTA was inhibitory at higher ratios.[38] 250 μM EDTA should therefore have been sufficient to account for available metal in F10 medium in our studies. DTPA, which forms more stable iron and copper chelates than EDTA[31] confirmed the findings obtained using EDTA, suggesting that inadequate metal chelation or pro-oxidant EDTA-iron interactions were not responsible for progressive oxidation.

It is possible that metals bound to cells were unavailable for chelation by EDTA but were still capable of reacting with CL-OOH, explaining the greater loss of CL-OOH with cells. However, metal-mediated oxidation of LDL involves binding of metals to LDL.[29] Consequently, metals responsible for LDL oxidation should have been in equilibrium in solution to bind LDL (and therefore be available to bind to EDTA), thus this explanation is unlikely.

Advanced cell-mediated LDL oxidation may be only partially metal-dependent. Alternative oxidizing systems such as peroxidases (e.g. myeloperoxidase and glutathione peroxidase) could be involved, however both are known to decrease in activity as monocytes differentiate into macrophages.[45] As our studies were performed using resident mouse peritoneal macrophages a role for these enzymes appears unlikely. Cell-generated peroxynitrite could theoretically mediate LDL oxidation by macrophages. However, nitric oxide production is not required for macrophage-mediated LDL oxidation,[46] and induction of cellular nitric oxide synthesis protects against LDL oxidation.[47] Lipoxygenases have been suggested to be involved in cell-mediated LDL oxidation,[10,48,49] although a number of studies have indicated that lipoxygenase inhibition by agents without radical-scavenging functions does not inhibit cell-mediated LDL oxidation.[27,50,51]

The greater efficacy of chelators at earlier compared with later times suggests that cell-mediated oxidation is likely to involve metals, but that chelation of metals is less effective at inhibiting oxidation after extensive propagation has occurred than if metals are chelated from the outset. This is most likely to be explained by a greater concentration of pre-formed lipid peroxyl and alkoxyl radicals at the time of EDTA addition.

Although all measured lipid components of LDL oxidation were affected by metal availability, the generation of 7-KC was particularly sensitive to EDTA, implying a major requirement for redox metals for its ongoing generation. Whether this reflects the non-specific, lesser oxidizability of cholesterol as compared to polyunsaturated fatty acyl chains,[52,53] or indicates a specific metal requirement is unclear. Regardless, these data suggest that 7-KC could be generated by cells in the arterial intima, and imply that active redox cycling of metals may well be involved in such a process.

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