Enhancement of macrophage survival and DNA synthesis by oxidized-low-density-lipoprotein (LDL)-derived lipids and by aggregates of lightly oxidized LDL

John A. HAMILTON^{*1}, Wendy JESSUP[†], Andrew J. BROWN[†] and Genevieve WHITTY^{*}

^{*}Arthritis and Inflammation Research Centre, University of Melbourne, Department of Medicine, The Royal Melbourne Hospital, Clinical Sciences Building, Royal Parade, Parkville, VIC 3050, Australia, and [†]Heart Research Institute, 145 Missenden Road, Camperdown, NSW 2050, Australia

Human atherosclerotic plaque contains a partially characterized range of normal and oxidized lipids formed mainly from free and esterified cholesterol and phospholipids, some of which can be located in macrophage-derived ‘foam’ cells. Oxidation of low-density lipoprotein (LDL) is often considered as an important event leading to subsequent foam-cell development, which may also include enhanced cell survival and/or proliferation. The active component(s) in oxidized LDL (ox.LDL) causing macrophage proliferation is debated. We report here that the lipid component of ox.LDL can promote macrophage survival and DNA synthesis, the latter response showing a synergistic effect in the presence of low concentrations of macrophage colony-stimulating factor. 7-Ketocholesterol showed some stimulation of macrophage DNA synthesis whereas hypochlorite-oxidized

(i.e. apolipoprotein B-oxidized) LDL did not. Plaque-derived lipids could enhance macrophage survival. It has not been proven that LDL in lesions is oxidized sufficiently to be the dominant source of sterols *in vivo* or to be able to induce macrophage growth *in vitro* or *in vivo*; it has been suggested that aggregation of modified LDL *in vivo* is an important step in the deposition of intracellular lipid. We found that aggregation of lightly oxidized LDL potentiated dramatically its ability to stimulate macrophage DNA synthesis, indicating that extensive oxidation of LDL is not required for this response *in vitro* and perhaps *in vivo*.

Key words: apoB, CSF-1/M-CSF, foam cell, oxysterol, plaque lipid.

INTRODUCTION

Cholesterol- and cholesterol ester-enriched macrophage-derived ‘foam’ cells are an early and characteristic feature of atherosclerotic lesions [1]. Human atherosclerotic plaque contains a partially characterized range of normal and oxidized lipids derived principally from non-esterified and esterified cholesterol and phospholipids [2]; many of the oxidized products are present at significant and potentially biologically active concentrations. Oxysterols, similar to those formed during low-density lipoprotein (LDL) oxidation (see below), are present [3,4] and are also in the foam cells [5]. Two of the predominant species are 7-ketocholesterol (7K) and 7 β -hydroxycholesterol (7 β OH), which are almost certainly formed by non-specific oxidation in the intima. It is particularly interesting that the oxysterol content (as percentage of cholesterol) of foam cells is 10-fold higher than that of total plaque tissue, suggesting that oxysterols are either generated or actively concentrated within these cells [2]. As well as oxysterols, several types of cholesterol ester in which the fatty acyl groups are oxidized have also been detected in human atheroma [4,6].

In vitro studies have shown that LDL can only induce cellular cholesterol accumulation when presented to macrophages in a modified form such that its uptake is mediated by either receptors other than the LDL receptor or phagocytic uptake, resulting in the progressive deposition of cholesterol, principally in esterified form, for example as cytoplasmic ‘foamy’ droplets within the cells. Oxidation has been identified as a modification that transforms LDL into such a high-uptake form [7]. When LDL undergoes oxidation *in vitro* a number of changes in lipid

composition occur, including a substantial loss of free and esterified cholesterol, and the concomitant generation of oxidation products of cholesterol and unsaturated fatty acyl esters of cholesterol [8]. Copper-catalysed oxidation of LDL provides a useful model system for studying the effects of oxidized LDL (ox.LDL) metabolism by macrophages. After 24 h of copper oxidation, cholesterol comprises approx. 50% of total sterols, 7K approx. 30%, with five other oxysterols (7 β OH and 7 α OH, cholesterol β - and β -epoxides, and 6 β -hydroxycholesterol) comprising most of the remainder [8]. In the earlier stages of oxidation 7-hydroperoxycholesterol is present, but this is converted to the other 7-oxysterols within 24 h [4]. Macrophage foam cells produced by uptake of 24 h-copper-ox.LDL display a remarkably similar profile of oxysterols to that of 24 h-copper-ox.LDL itself [8,9].

During LDL oxidation, fatty acyl chains of sterol esters (which comprise about 70% of cholesterol in native LDL and about the same proportion of all oxysterols in ox.LDL) also undergo oxidation [4,8]. Early products include fatty acid hydroperoxides, hydroxides and ketones. These are present in significant amounts in human atherosclerotic plaque [4,6,10]. For example, cholesterol linoleate hydroperoxide and hydroxide comprise up to 30% of total fatty acyl ester in some cases. In copper-ox.LDL-loaded macrophages the majority of the cholesterol esters and 7K esters also contain fatty acyl chains that are presumed to be oxidized [8], but most of these are more-advanced oxidation products than those listed above.

Aggregation is a characteristic of extensively oxidized LDL [11,12] and, as well as the chemical modification, is believed to contribute to the poor processing in macrophages [12]. LDL

Abbreviations used: LDL, low-density lipoprotein; ox.LDL, oxidized LDL; ac.LDL, acetylated LDL; 7K, 7-ketocholesterol (= 7-oxocholesterol); 7 β OH, 7 β -hydroxycholesterol; BMM, bone-marrow-derived macrophage; CSF-1, macrophage colony-stimulating factor; apoB, apolipoprotein B; LPDS, lipoprotein-deficient human serum; TdR, thymidine; AAPH, 2,2'-azobis-(2-amidinopropane) hydrochloride; REM, relative electrophoretic mobility.

¹ To whom correspondence should be addressed (e-mail jahami@unimelb.edu.au).

aggregation also appears to occur in human atherosclerotic plaque [13], although several different processes may contribute to lesion lipoprotein aggregation in addition to oxidation (see [14] for review). It has been suggested that aggregation of modified LDL *in vivo* is an important step in the deposition of intracellular lipid [12,13].

There are a number of reports showing that macrophage-derived foam cells can proliferate in both human and animal lesions, particularly in the early phase of the disease [15–20]. We reported that treatment of murine bone-marrow-derived macrophages (BMMs) with low doses of ox.LDL, but not with non-modified LDL, led to cell survival, DNA synthesis, and an enhanced proliferative response to macrophage colony-stimulating factor (CSF-1 or M-CSF) and granulocyte macrophage CSF [21]; subsequently we showed a similar potentiation of human monocyte survival [22]. It was suggested that modified LDL could maintain foam-cell survival, thereby lengthening their tenure in the plaque, as well as 'priming' them so that they could proliferate better in response to local concentrations of these CSFs [21,22]. There is debate regarding the components of ox.LDL that are responsible for the stimulation of macrophage DNA synthesis. In some studies, the mitogenic effect was reported to be mediated by protein kinase C and was attributed to lysophosphatidylcholine, which is generated during LDL oxidation [23,24]. In contrast, it was recently proposed that modification of apolipoprotein B (apoB) accounts for most of the induction of macrophage growth by extensively oxidized LDL and that lysophosphatidylcholine was not required [25].

In this study we show that the lipid component of ox.LDL can promote BMM survival and DNA synthesis, including a synergistic effect on DNA synthesis with a low dose of CSF-1. 7K had some activity, suggesting that it may be contributing. We also demonstrate that aggregation of lightly oxidized LDL increases its ability to stimulate BMM DNA synthesis. Finally, lipids extracted from some human atheroma promoted macrophage survival.

MATERIALS AND METHODS

Mice

Cells were obtained from male or female CBA mice as described before [21].

BMMs

BMMs were generated as adherent cells from their non-adherent progenitors in bone marrow as described in [21] and grown to confluence in 24-well plates (Nunc) for 5–6 days in RPMI 1640 supplemented with 20 mM Hepes, 15% (v/v) fetal calf serum and 20% (v/v) L-cell-conditioned medium (a crude source of CSF-1). The BMMs are a relatively pure and homogeneous population with $\geq 95\%$ of the adherent cells binding CSF-1 [26]. Cells were usually prepared for experiments by washing twice with PBS and either the experiment was commenced immediately or the cells were incubated for 24 h in growth medium without L-cell-conditioned medium to render them quiescent before commencement [21]. Dilutions of LDLs were made in RPMI 1640/10% (v/v) endotoxin-free lipoprotein-deficient human serum (LPDS).

DNA synthesis

DNA synthesis was measured as the incorporation of [*methyl*- ^3H]thymidine (TdR; 2 $\mu\text{Ci/ml}$) [21]. Uptake was stopped by

removal of the culture medium and solubilization in 0.2 M NaOH, and the incorporation of label into trichloroacetic acid-precipitable material was recovered using an Inotech cell harvester (Berthold, Melbourne, Australia). Incorporated radioactivity was counted on a scintillation counter.

BMM numbers

For quantification of the number of BMMs in culture, the medium was removed and the cells gently scraped; viable cells were counted in a haemocytometer with the use of Trypan Blue exclusion [21].

LDL preparation

Human LDL was isolated from healthy, fasting volunteers in the presence of 3 mM EDTA by discontinuous KBr density-gradient ultracentrifugation in the density range $\rho = 1.02\text{--}1.05$, as described previously [8]. The isolated LDL was sterilized by membrane filtration (0.45 μm) and stored in the dark at 4 °C under N_2 . LDL preparations were used within 1 week of isolation. All materials and solutions were pretreated to remove endotoxin, and LDL isolation was under sterile conditions.

LDL modifications

Oxidation and acetylation

LDL was desalted into PBS by passage over two Sephadex G-25 columns (PD-10; Pharmacia) in series to remove KBr and EDTA. Copper-induced oxidation was achieved by incubating LDL (1 mg of protein/ml) in PBS (minus Ca^{2+} and Mg^{2+}) with a sterile solution of CuCl_2 (final concentration, 20 μM) at 37 °C for 24 h, unless otherwise indicated [8]. Oxidation was arrested by addition of serum or by dialysis against PBS/EDTA to remove excess CuCl_2 , and the material was used within 7–14 days of preparation.

LDL (3–4 mg of protein/ml) was usually acetylated as described in [27]; a comparison was made with material produced according to [25]. Excess reagents were removed by gel filtration as described above.

The degrees of acetylation and oxidation were assessed by non-denaturing gel electrophoresis on 1% Universal agarose gels (Ciba-Corning) in Tris-barbitone buffer (pH 8.6) at 90 V for 45 min [27]. The LDL band was visualized with Fast Red 7B stain. A relative electrophoretic mobility (REM) of 2–3, with native LDL as a reference (REM = 1), was routine.

ApoB oxidation

LDL was incubated with a 400-fold molar excess of hypochlorite [28] immediately before use; the incubation was for 15 min on ice with LDL at 1 mg/ml. A REM value of 2.5–3.5 was obtained routinely [28].

Oxysterols and fatty acid oxidation products

7K and 7 β OH were incorporated into native LDL as described previously [29]. Briefly, human plasma was incubated with the appropriate oxysterol, at a final concentration of 2.4 mM, for 6 h at 37 °C with gentle mixing. LDLs enriched by oxysterols (by 7K or 7 β OH) were then isolated as described above. Where required, the lipoproteins were subsequently acetylated as described above (to produce 7K-ac.LDL or 7 β OH-ac.LDL, where ac.LDL is acetylated LDL). We have shown previously that the incorporated oxysterols remain stably associated with LDL throughout isolation and acetylation [29].

Treatment of ac.LDL with the water-soluble peroxy-lipid generator, 2,2'-azobis-(2-amidinopropane)-hydrochloride (AAPH), leads to relatively selective generation of lipid hydroperoxides, of which cholesterol linoleate hydroperoxide is quantitatively the major product [30]. The reaction was terminated by gel filtration to remove AAPH and, in the absence of metals, this resulting hydroperoxide-enriched LDL is stable. Stoichiometric conversion of hydroperoxides into hydroxides is achieved by incubation of this modified LDL with Ebselen and glutathione [30]. These protocols permit formation of ac.LDL selectively enriched with cholesterol ester hydroperoxides (ac.LDL-OOH) or hydroxides (ac.LDL-OH) in the absence of the other major lipid-oxidation products generated during copper oxidation of LDL. In particular, few oxysterols are generated during this procedure.

Aggregation

LDLs (3 mg of protein/ml) were vortexed at room temperature for 15 s as a published procedure for aggregation [12,31]. Turbidity was noticed following this procedure.

Lipid extraction from native LDL and ox.LDL

Lipids were extracted from the respective LDLs by Folch extraction [32], evaporated to dryness and resuspended (equivalent to 2 mg of LDL protein/ml) in 100 % LPDS in a sonicating water bath.

Atherosclerotic artery samples

Samples of human atherosclerotic lesions were obtained from six patients, undergoing a carotid endarterectomy. Five of the patients were male and all patients ranged in age from 57 to 75 years. This study was approved by the Central Sydney Area Health Service Ethics Review Committee and full and informed written consent was obtained from the patients. Within 1 h of excision, samples were placed into Chelex-treated and argon-flushed PBS containing EDTA (1 mM), butylated hydroxytoluene (0.1 mM), penicillin (100 units/ml) and streptomycin (100 µg/ml). Samples were freeze-dried and extracted with acetone [4]. For subsequent delivery to macrophages, the lipid extract was evaporated to dryness and resuspended (2 mg of total cholesterol/ml) in 100 % LPDS in a sonicating water bath. Samples were diluted into culture medium.

Lipid measurement

Sterols and sterol esters were extracted from native and modified lipoproteins and from cell lysates into n-hexane, as described previously [33]. Lesion lipid extracts (described above) were subjected to ice-cold alkaline saponification as detailed previously, using 19-hydroxycholesterol as an internal standard [4]. Cholesterol and cholesterol esters were separated using reversed-phase HPLC at room temperature on a C-18 column (Supelco), using an eluent of acetonitrile/isopropanol (propan-2-ol) (30:70, v/v) and detection at 210 nm [33]. Free and esterified 7K, cholesterol linoleate hydroperoxide and cholesterol linoleate hydroxide were analysed with a solvent system of acetonitrile/isopropanol/water (44:54:2, by vol.) and detection at 234 nm [8,33]. Other oxysterols were determined after sample saponification and involved normal-phase HPLC with detection at 234 nm [4]. Separation was achieved by an Alltima Silica column (Alltech; 0.46 cm × 15 cm, 10 µm) with a 3 cm guard column (Ultremex; 3 µm particle size; Phenomenex, Australian Chromatography Co., Thornleigh, NSW, Australia) and a mobile phase of hexane/isopropanol/acetonitrile (94.8/4.6/0.6, by vol.)

run at a flow rate of 1.0 ml/min. The mobile phase was degassed thoroughly by sonication under vacuum immediately prior to use and was sparged continually with helium during HPLC analysis. Typical retention times of 7K, 19-hydroxycholesterol and 7βOH were 15.2, 23.9 and 24.9 min respectively [4].

Reagents

The following reagents were obtained from commercial sources: [³H]TdR (80 Ci/mmol; Amersham), fetal calf serum (CSL, Parkville, Australia) and AAPH (Polysciences, Warrington, PA, U.S.A.). Recombinant human CSF-1 (M-CSF), which is biologically cross-reactive on murine cells, was a gift from Chiron Corp., Emeryville, CA, U.S.A.

All other reagents were of analytical grade. All practical precautions for minimizing endotoxin contamination were taken. Solutions were made in pyrogen-free water (Delta West), and endotoxin levels were monitored routinely by Limulus lysate tests (CSL), with the minimum detectable level being 10 pg/ml [21].

RESULTS

Comparison of the effects of ox.LDL and ac.LDL on BMM DNA synthesis

As part of the analysis to determine what might be the active component(s) in modified LDL and to explore further at the cellular level how BMM survival and DNA synthesis are promoted, we first compared the relative changes induced in BMM by ox.LDL and ac.LDL. We stated before [21] that ac.LDL (50 µg of protein/ml), in addition to ox.LDL, could stimulate BMM survival and also DNA synthesis. We have now compared the relative abilities of the respective modified LDLs to stimulate BMM DNA synthesis, in both the absence and presence of CSF-1. As before, with ox.LDL [21] and now with ac.LDL, the BMMs, which normally die by apoptosis in the absence of CSF-1 [34], spread on the tissue-culture surface and stayed alive. When DNA synthesis was quantified by [³H]TdR incorporation, the dose-response curves were similar, with both modified LDLs being active at ≥ 3 µg of protein/ml (Figure 1);

Figure 1 Effect of ac.LDL and ox.LDL on BMM DNA synthesis

BMMs, from which CSF-1 had just been removed, were treated with increasing concentrations of ac.LDL (●, ○) or ox.LDL (■, □), both in the absence (○, □) or presence (●, ■) of 160 units/ml CSF-1. For this particular experiment, after 24 h of incubation with lipoproteins, the cultures were washed twice with PBS and either left untreated or treated again with CSF-1 daily. The cultures were then pulsed with [³H]TdR and harvested 48 h later. Data are means ± S.E.M. from triplicate cultures from a representative experiment that was repeated a total of five times.

Figure 2 Effect of ox.LDL lipids on BMM DNA synthesis

BMMs, from which CSF-1 had just been removed, were treated with increasing concentrations of lipids extracted from ox.LDL (●, ○) or intact ox.LDL (■, □; see the Materials and methods section), in the absence (○, □) or presence (●, ■) of CSF-1 (320 units/ml), added daily. The cultures were pulsed with [3 H]TdR at 24 h and harvested 48 h later. The concentrations of the lipids in the starting sample were based on the amount of protein present in ox.LDL, assuming complete lipid extraction. Data are means $\pm$ S.E.M. from triplicate cultures from a representative experiment that was repeated five times.

also, both materials gave synergistic effects with a suboptimal CSF-1 concentration, again with similar dose-response curves (Figure 1). In the particular experiment shown, the maximum ac.LDL effect (at 50 μ g of protein/ml) was less than the maximum ox.LDL effect (approx. 30–40%); this relative effect was generally observed, but in other experiments with different preparations the degree of stimulation of DNA synthesis due to an optimal ac.LDL concentration was as high as 60% of that achieved with an optimal ox.LDL concentration. As the method for LDL acetylation used in a separate study, in which ac.LDL was ineffective in stimulation of macrophage DNA synthesis [25], was slightly different to our protocol [27], we compared the relative efficacies of LDLs acetylated by these two procedures. The REMs (see the Materials and methods section), a rough index of the degree of acetylation, were the same for both preparations. In three independent experiments the two ac.LDL samples (50 μ g of protein/ml) had similar effects on BMM DNA synthesis (results not shown); dose-response curves were also similar.

Subsequent to uptake, endocytosed lipoproteins are degraded in the lysosomal system. It was shown previously that the relative lysosomal degradation rates of ac.LDL and ox.LDL in murine peritoneal macrophages are very different; ac.LDL is cleared rapidly from the lysosomes within 4 h, whereas some ox.LDL components are resistant to lysosomal hydrolysis and persist in this compartment for days [35,36]. If lysosomal storage is a signal for DNA synthesis, then the ac.LDL effect on BMM DNA synthesis might be expected to be more short-lived than that of ox.LDL. However, in preliminary experiments where the respective modified LDLs were added overnight, the media then removed and replaced with lipoprotein-free medium and DNA synthesis measured 3 days later, the relative degree of DNA synthesis was similar to what was found above in the shorter experiments and was maintained (results not shown); these data suggest that a persistent lysosomal presence is not an essential feature.

Another parameter that could be governing the extent of the BMM response to modified LDL is the cholesterol loading. We found in BMMs that ox.LDL treatment led to lower accumulation of cholesterol ester and free cholesterol than treatment with

equivalent concentrations of ac.LDL (results not shown). We have shown previously that ox.LDL-loaded macrophages contain a substantial lysosomal pool of undegraded sterol esters, in which the sterol component is predominantly cholesterol or 7K, and the fatty acid is highly oxidized [9]. Although we could find no correlation between total cholesterol loading and the extent of BMM DNA synthesis, it remains possible that the presence of undegraded ox.LDL components in the lysosomal compartment may contribute to the increased DNA synthesis observed in ox.LDL-loaded macrophages compared with their ac.LDL-loaded counterparts.

Ox.LDL-derived lipids and macrophage DNA synthesis

We determined whether any of the activities responsible for enhanced BMM DNA synthesis reside in the lipids. As can be seen in Figure 2, a dose-dependent increase in BMM DNA synthesis was observed in BMMs treated with the lipids extracted from ox.LDL. This effect was also monitored in the presence of a suboptimal CSF-1 concentration (320 units/ml). Ox.LDL lipids were as active as comparable concentrations of whole ox.LDL in the presence of CSF-1 and even in its absence promoted DNA synthesis to approx. 60% of values with whole ox.LDL (Figure 2). It has been reported previously in macrophages incubated with 24 h-copper-ox.LDL that the cellular enrichment in oxysterols displayed a remarkably similar profile to that found in the 24 h-copper-ox.LDL itself [8,33]. Likewise, we found that the 7K content was similar for BMMs loaded with the ox.LDL-derived lipids or with ox.LDL itself (results not shown). Also, measurement of the cellular content of total cholesterol (cholesterol esters and free cholesterol) and oxidized lipids after exposure to ox.LDL or ox.LDL-derived lipids confirmed that similar loadings were achieved by both agents. For example, in one experiment representative of three, after a 24 h incubation with 50 μ g of protein/ml ox.LDL or an equivalent concentration of ox.LDL-derived lipids, the levels of cholesterol and 7K were 95 and 6.4 nmol/culture and 95 and 7.4 nmol/culture, respectively. The lipids extracted from native LDL, as was found before with the whole native LDL [21], were inactive over the concentration range 3–100 μ g of protein/ml (results not shown).

Oxysterols and BMM DNA synthesis

Given the data in Figure 2 we next determined the activity of some of the components known to be in the lipid component of ox.LDL. In the ox.LDL used in this study, approx. 50% of the lipoprotein cholesterol (both free and esterified) was converted to oxysterols. Approx. 30% of total sterol was oxidized to 7K, with five other oxysterols, including 7 β OH, comprising the remainder [8]. In an earlier study, the kinetics of 7K generation measured during the timed copper-catalysed oxidation of LDL matched the ability of the ox.LDL to stimulate BMM DNA synthesis [21]. Therefore, we next determined whether 7K, and also the structurally related 7 β OH, were effective in our system. The oxysterols were delivered to the macrophages incorporated into LDL or ac.LDL [29]. This avoids any problems of toxicity that have been reported by some groups after direct addition of oxysterols to cells (from ethanolic solution) [37]. We show in Figure 3 that 7K, and, to a lesser extent, 7 β OH, had a mild stimulatory effect on BMM DNA synthesis when administered with LDL, although the effect was far less than that for ox.LDL; however, under these conditions the amounts of individual oxysterols present in the cells were similar to those achieved by uptake of ox.LDL, i.e. they did not correlate with the extent of the DNA-synthesis response (results not shown).

Figure 3 Effect of oxysterols on macrophage DNA synthesis

BMMs, from which CSF-1 had just been removed, were untreated or treated with 50 µg of protein/ml of LDL, 7K-enriched LDL (7K-LDL), 7βOH-enriched LDL (7βOH-LDL) or ox.LDL for 24 h, and then removed. The cultures were then pulsed with [³H]TdR and harvested 48 h later. Data are means ± S.E.M. from triplicate cultures from a representative experiment that was repeated twice more. **P* = 0.002; ***P* < 0.001, compared with the untreated group (Student's *t* test).

Figure 4 Effect of 7K delivery via ac.LDL on BMM DNA synthesis

BMMs, from which CSF-1 had just been removed, were treated with increasing concentrations of ac.LDL (○) or 7K-enriched ac.LDL (□). For this particular experiment, at 24 h the cultures were washed twice with PBS and RPMI 1640/10% fetal calf serum added. The cultures were pulsed with [³H]TdR and harvested 48 h later. Data are means ± S.E.M. from triplicate cultures from a representative experiment that was repeated a total of six times. Error bars were omitted when they were smaller than the symbol.

7K was also active if delivered via ac.LDL. Under these conditions the cells become heavily loaded with cholesterol (i.e. foam cells) as well as enriched with 7K [29]. A dose-response curve to this formulation is given in Figure 4, with the background effect of ac.LDL included. Once again, synergistic effects were noted when 7K was added with a suboptimal CSF-1 concentration (160 units/ml; results not shown).

Cholesterol ester peroxide, cholesterol ester hydroxide and macrophage DNA synthesis

During LDL oxidation, fatty acyl chains of sterol esters also undergo oxidation. Some of the known products are fatty acid hydroperoxides, hydroxides and ketones [8,33]; these have been detected in human lesions in quite significant proportions [10]. Because of this, although these compounds are only minor components of the ox.LDL used in the present study, we determined their effects on macrophage DNA synthesis. To

Table 1 Aggregation of ox.LDL and macrophage DNA synthesis

BMMs, from which CSF-1 had been removed 24 h previously, were either left untreated or treated with 50 µg of protein/ml ox.LDL or aggregated ox.LDL (see the Materials and methods section), in the absence or presence of CSF-1 (160 units/ml) added daily. Cultures were pulsed with [³H]TdR at 24 h and harvested 48 h later. Data are means ± S.E.M. from triplicate cultures from a representative experiment, which was repeated 12 times.

Addition	10 ⁻⁴ × [³ H]TdR incorporation (c.p.m.)	
	– CSF-1	+ CSF-1
None	0.2 ± 0.02	2.2 ± 0.1
ox.LDL	6.3 ± 0.2	12.1 ± 1.6
Aggregated ox.LDL	12.4 ± 0.3	22.2 ± 0.8

selectively study the effects of cholesterol ester oxidation products on macrophage function, ac.LDL was subjected to mild oxidation using the peroxy radical generator, AAPH (see the Materials and methods section). Under the conditions used, cholesterol ester hydroperoxides (ac.LDL-OOH) are generated in the particle without significant oxidation of other LDL constituents such as α-tocopherol, cholesterol or apoB [30]. Reduction of ac.LDL-OOH with Ebselen and glutathione converts the hydroperoxides stoichiometrically to hydroxides (ac.LDL-OH). The relatively mild oxidation that ac.LDL undergoes to generate these products does not affect its affinity for the scavenger receptor, so that ac.LDL-OOH or ac.LDL-OH are readily taken up by the cells [30]. In three independent experiments, neither hydroperoxide- nor hydroxide-enriched ac.LDL potentiated the effect of ac.LDL (results not shown). This lack of effect was also noted over the concentration range 3–100 µg of protein/ml (results not shown); the peroxide content in the ac.LDL-OOH used was 167 ± 3 nmol/mg of LDL protein, representing oxidation of approx. 30% of the cholesterol esters.

Oxidized apoB and macrophage DNA synthesis

We next tested whether direct oxidative modification of apoB by hypochlorite could also lead to BMM DNA synthesis. In three independent experiments we found that this method of oxidation failed to convert LDL into a form capable of inducing DNA synthesis over the concentration range 2–100 µg of protein/ml and also failed to potentiate the DNA synthesis due to low CSF-1 concentration (results not shown); however, at 50 µg of protein/ml it could partially prevent the loss of BMMs by apoptosis following CSF-1 removal [34], indicating a weak response.

Aggregated ox.LDL and macrophage DNA synthesis

We next compared the effect of aggregation on the activity of ox.LDL (see the Materials and methods section). We see in Table 1 that aggregation of ox.LDL potentiated its stimulation of BMM DNA synthesis in both the absence and presence of a suboptimal CSF-1 concentration. This enhancement due to aggregation was manifested at all ox.LDL doses tested (3–50 µg of protein/ml), with toxic effects again being noted at 100 µg of protein/ml in most experiments [21]. In the experiment whose data are provided in Table 1 the ox.LDL samples were removed after 24 h and the cells pulsed with [³H]TdR for a further 48 h. Similar results, however, were obtained if ox.LDL samples were left on the macrophages for the entire 72 h.

We showed previously that 2–4 h of incubation during copper-induced oxidation was required before LDL was oxidized

Figure 5 Effect of aggregation of lightly oxidized LDL on BMM DNA synthesis

BMMs, from which CSF-1 had been removed 24 h previously, were untreated or treated with LDL (50 $\mu\text{g}/\text{ml}$) that had been oxidized for different times in the (a) absence (○) or (b) presence (●) of 160 units/ml CSF-1. The cells were also treated with the same modified LDL samples that had also been aggregated (see the Materials and methods section), in the (a) absence (□) or (b) presence (■) of 160 units/ml CSF-1. For this particular experiment, at 24 h the cultures were washed twice with PBS and left untreated or treated again with CSF-1 daily. The cultures were pulsed with [^3H]TdR and harvested 48 h later. Data are means $\pm$ S.E.M. from a representative experiment that was repeated three times. Error bars were omitted when they were smaller than the symbol.

sufficiently to begin to enhance BMM DNA synthesis, the effect increasing with an oxidation time up to 24 h [21]. We determined whether aggregation would potentiate the activity of the relatively lightly oxidized LDL produced after short incubation times. We see in Figure 5 that this is so, both in the absence (Figure 5a) and presence (Figure 5b) of a suboptimal CSF-1 concentration. With some batches, aggregation could even convert native LDL (0 h oxidation) into a slightly active form for promoting survival and even a small degree of DNA synthesis. Aggregation increased uptake of native and lightly oxidized (0–4 h oxidation) LDL, which was reflected in the higher cell content of total cholesterol and oxidized lipids after exposure to the aggregated lipoproteins (results not shown). However, once again there was no direct correlation with the extent of DNA synthesis (results not shown).

Other lipoprotein types, namely high-density lipoproteins HDL₂ and HDL₃, also gave similar results to LDL, in that their oxidized forms, particularly when aggregated, stimulated BMM DNA synthesis and potentiated the action of low doses of CSF-1 (results not shown).

Effect of plaque lipids

To see whether our *in vitro* model system may have some clinical relevance, we tested the effect of lipids isolated from the plaques

Table 2 Effect of atherosclerotic plaque lipids on BMM survival

BMMs, from which CSF-1 had just been removed, were counted [$(75 \pm 4) \times 10^3$ cells]. They were then either left untreated or treated with extracted carotid plaque lipids (25 μg of total sterol/ml) from six patients (see the Materials and methods section). After 72 h, cell number was counted in a haemocytometer. The control group represents cells exposed to a 'mock' extraction and resuspension of lipid to make allowance for any extraction solvent introduced into the system. Data are means $\pm$ S.E.M. from triplicate cultures from a representative experiment that was repeated twice. The respective plaque lipid contents of the following oxysterols, measured as a percentage of the total cholesterol, are also provided: 7K, 27-hydroxycholesterol (27OH), 7 α -hydroxycholesterol (7 α OH) and 7 β OH. * $P < 0.001$; † $P = 0.02$, compared with the control group (Student's *t* test).

Plaque no.	10 ⁻³ $\times$ Cell number	Oxysterol content (% total cholesterol)			
		7K	27OH	7 α OH	7 β OH
—	18 $\pm$ 1	—	—	—	—
P1	52 $\pm$ 3*	0.55	0.1	0.05	0.06
P2	33 $\pm$ 1*	1.9	0.1	0.05	0.03
P3	57 $\pm$ 6*	0.92	0.14	0.11	0.05
P4	28 $\pm$ 3†	1.06	0.06	0.06	0.03
P5	17 $\pm$ 2	0.89	0.1	0.07	0.05
P6	22 $\pm$ 2	0.88	0.12	0.05	0.04
Control	22 $\pm$ 2	—	—	—	—

of patients with atherosclerosis. As presented in Table 2 such lipids from four out of the six patients screened were able to promote BMM survival. As can be seen from Table 2 there was no obvious relationship between the oxysterol content of the lipids (expressed as a percentage of the total cholesterol content) and their ability to enhance cell survival. When DNA synthesis was measured there was a slight stimulation by one of the samples (P3; results not shown).

DISCUSSION

We showed before that ox.LDL could promote BMM survival and DNA synthesis [21]; in that report it was suggested that macrophage responses to ox.LDL should also be monitored in the presence of CSF-1, since this factor normally circulates at what are considered to be at least 'survival' doses for cells of the macrophage lineage [38]. With this approach, we found [21] that low doses of CSF-1 could become mitogenic for ox.LDL-loaded BMMs and that there was a synergistic proliferative response in the presence of the two stimuli. We now show that lipids extracted from ox.LDL, but not from native LDL, were quite active at promoting BMM survival and DNA synthesis, the latter response again being potentiated in the presence of low CSF-1 concentrations. It has been shown previously that the binding of ox.LDL to murine resident peritoneal macrophages can be inhibited (up to 70 %) by the apoB isolated from ox.LDL, indicating that macrophage recognition of ox.LDL is primarily dependent on its modified protein moiety [39]. However, additional experiments have suggested that the lipid moiety plays a role in the ox.LDL interaction with macrophage receptors [39,40]. Our observations described here are consistent with such a contribution from oxidized lipids to macrophage recognition of ox.LDL and hence to cellular responses. However, it is also possible that the stimulatory oxidized lipids that we have identified in the present study interact with and enter the cell by quite distinct mechanisms from those by which ox.LDL operates. Other studies have claimed that the mitogenic activity of ox.LDL on murine peritoneal macrophages is due to lysophosphatidylcholine, generated during LDL oxidation, and is through protein kinase C activation [23,24]. However, the requirement for lyso-

phosphatidylcholine was not supported in an independent study, where modification of apoB by derivitization with lipid peroxidation products was proposed to account for most of the induction of macrophage growth by extensively oxidized LDL [25]. In our hands, direct modification of apoB in LDL by hypochlorite, even though converted into a high-uptake form for macrophages [28], failed to induce BMM DNA-synthesis-stimulating activity.

The disparity between the sterol profiles of ox.LDL-loaded macrophages and human foam cells suggests that ox.LDL, i.e. an extensively oxidized form(s), is not the dominant source of sterols *in vivo* [8,41]. Specifically, while oxysterols are present in lesion foam cells, they comprise only a few percent of total sterol, unlike macrophages loaded with ox.LDL *in vitro*, where oxysterols represent up to 50% of the total sterol content. Because oxysterols, such as 7K, are abundant in extensively oxidized LDL [8], because we had found before that the effectiveness on the BMMs of ox.LDL with different degrees of oxidation increased, as did its 7K content [21], and because such oxysterols are present in human atherosclerotic plaques and foam cells [6], we examined their effects of BMMs. Some studies have examined the effects of specific oxysterols on macrophage function by incubation of macrophages with free oxysterols added directly to the culture medium [37]. In order to avoid the very high intracellular concentrations resulting from the direct addition of free 7K, a more physiological method of delivering oxysterols has been utilized, i.e. incorporation into lipoprotein. This method uses a combination of ac.LDL (the lipoprotein is endocytosed by the scavenger receptor but the lipoprotein lipids are not oxidized) and 7K-enriched ac.LDL. By controlling the amount of oxysterol-enriched ac.LDL it is possible to produce a cell whose sterol content more closely resembles the authentic cell type [2]. Using such an approach we found that 7K administered with ac.LDL (or LDL) behaved, to some extent at least, like ox.LDL and its extracted lipids, in that it could promote some degree of BMM DNA synthesis; thus oxysterols may form part, but by no means all, of the ox.LDL stimulatory activity in our system, and may contribute to the generally higher stimulatory activity of ox.LDL than ac.LDL (see above). However, following BMM 'loading' with various agents, no direct correlation could be found between 7K content and the extent of DNA synthesis. We could find no evidence for a contribution from cholesterol ester peroxide or hydroxide.

As mentioned, there is evidence suggesting that heavily oxidized LDL may not be the material responsible for foam-cell formation in plaques and that the ox.LDL-loaded macrophage is an incomplete *in vitro* foam-cell model [41]; also, it has not been proven that LDL in early or developing lesions in humans is oxidized to the extent that it can induce macrophage growth induction *in vitro* or *in vivo* [13]. It has been suggested that aggregation of modified LDL *in vivo* is an important step in the deposition of intracellular lipid [11,42]; such aggregation has been observed in plaques [12,43]. We showed here that ox.LDL with a low degree of oxidation (i.e. produced by a short oxidation time in the presence of Cu²⁺ [21]), when aggregated prior to culture, became quite potent, with its effect on DNA synthesis being at least comparable with or even better than the response to extensively oxidized LDL (Figure 5). Such aggregation offers a mechanism for weakly oxidized LDL to become more potent, with possible significance given the evidence for such mild oxidation in human plaques referred to earlier [13,25,41]. Whether spontaneous aggregation of any of the other materials, shown to be active on the BMM either here or previously [21], was occurring during the culture period, and thereby contributing, remains to be established; it has been concluded by

others that aggregation of *in vitro*-modified LDL is a necessary step in intracellular lipid deposition [11].

It was observed above that lipids isolated from the plaques of some donors could promote BMM survival and, in one case, induce DNA synthesis. This observation was similar to that found with aggregated LDL (or low-dose CSF-1), i.e. predominantly cell survival with a weak DNA-synthesis response. We have proposed before that enhancement of macrophage survival by modified LDL can also be significant in itself since, if occurring in lesions, it would lead to increased numbers of foam cells capable of contributing to the progression of the atherosclerotic lesion; in other words, foam-cell proliferation may not be necessary [21,22]. It has been reported that atherosclerotic lesion-derived LDL undergoes aggregation at sufficiently high concentration *in vitro*, and accelerated uptake by macrophages of LDL isolated from the arterial intima can be attributed largely to phagocytosis of LDL-containing aggregates, with oxidation *in vivo* possibly contributing to this aggregation [43]. It would be of interest to assess the actions of plaque lipids following experimental aggregation *in vitro* as we have done with ox.LDL.

We demonstrated in this paper that ac.LDL was quite potent at promoting BMM DNA synthesis. This observation is different from that made with resident murine peritoneal macrophages [23,25,44], but similar to the findings in one of these papers with peritoneal exudate macrophages [44]. Uptake of ox.LDL by macrophages occurs in part by way of the ac.LDL receptor [45–47]. However, as much as 30–70% of ox.LDL uptake by murine peritoneal macrophages is not competitively inhibited by ac.LDL [46,47] and several lines of evidence point to the existence of a number of receptors for ox.LDL [45]. The contribution of this different receptor usage to the relative effects on BMM DNA synthesis remains to be elucidated. In agreement with a prior study with peritoneal exudate macrophages [44], we could find no correlation with the cholesterol ester loading resulting from respective treatments of BMM with ox.LDL and ac.LDL and the resultant level of DNA synthesis.

In summary, we have reported here that ox.LDL-derived lipids, in contrast to oxidized apoB, promoted BMM survival and DNA synthesis; 7K showed some activity. Aggregation of lightly oxidized LDL potentiated its activity; plaque lipids could enhance survival. The mode of action(s) of the various stimuli and *in vivo* relevance remain to be determined.

This work was supported by grants from the National Health and Medical Research Council of Australia and the National Heart Foundation of Australia. We thank R. Sallay for typing the manuscript.

REFERENCES

- Ross, R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature (London)* **362**, 801–809.
- Brown, A. J. and Jessup, W. (1999) Oxysterols and atherosclerosis. *Atherosclerosis* **142**, 1–28.
- Carpenter, K. L., Taylor, S. E., Ballantine, J. A., Fussell, B., Halliwell, B. and Mitchinson, M. J. (1993) Lipids and oxidised lipids in human atheroma and normal aorta. *Biochim. Biophys. Acta* **1167**, 121–130.
- Brown, A. J., Leong, S. L., Dean, R. T. and Jessup, W. (1997) 7-Hydroperoxy-cholesterol and its products in oxidized low density lipoprotein and human atherosclerotic plaque. *J. Lipid Res.* **38**, 1730–1745.
- Hultén, L. M., Lindmark, H., Diczfalusy, U., Björkhem, I., Ottosson, M., Liu, Y., Bondjers, G. and Wiklund, O. (1996) Oxysterols present in atherosclerotic tissue decrease the expression of lipoprotein lipase messenger RNA in human monocyte-derived macrophages. *J. Clin. Invest.* **97**, 461–468.
- Suarna, C., Dean, R. T., May, J. and Stocker, R. (1995) Human atherosclerotic plaque contains both oxidized lipids and relatively large amounts of alpha-tocopherol and ascorbate. *Arterioscler. Thromb. Vasc. Biol.* **15**, 1616–1624.

- 7 Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. and Witztum, J. L. (1989) Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320**, 915–924
- 8 Brown, A. J., Dean, R. T. and Jessup, W. (1996) Free and esterified oxysterol: formation during copper-oxidation of low density lipoprotein and uptake by macrophages. *J. Lipid Res.* **37**, 320–335
- 9 Brown, A. J., Mander, E. L., Gelissen, I. C., Kritharides, L., Dean, R. T. and Jessup, W. (2000) Cholesterol and oxysterol metabolism and subcellular distribution in macrophage foam cells. Accumulation of oxidized esters in lysosomes. *J. Lipid Res.* **41**, 226–237
- 10 Niu, X., Zammit, V., Dean, R., May, J. and Stocker, R. (1999) Co-existence of oxidized lipids and α -tocopherol in all lipoprotein fractions isolated from advanced human atherosclerotic plaques. *Arterioscler. Thromb. Vasc. Biol.* **19**, 1708–1718
- 11 Tertov, V. V., Orekhov, A. N., Sobenin, I. A., Gabbasov, Z. A., Popov, E. G., Yaroslavov, A. A. and Smirnov, V. N. (1992) Three types of naturally occurring modified lipoproteins induce intracellular lipid accumulation due to lipoprotein aggregation. *Circ. Res.* **71**, 218–228
- 12 Hoff, H. F., Zyromski, N., Armstrong, D. and O'Neil, J. (1993) Aggregation as well as chemical modification of LDL during oxidation is responsible for poor processing in macrophages. *J. Lipid Res.* **34**, 1919–1929
- 13 Steinbrecher, U. P. and Loughheed, M. (1992) Scavenger receptor-independent stimulation of cholesterol esterification in macrophages by low density lipoprotein extracted from human aortic intima. *Arterioscler. Thromb. Vasc. Biol.* **12**, 608–625
- 14 Jessup, W. and Kritharides, L. (2000) Metabolism of oxidized LDL by macrophages. *Curr. Opin. Lipidol.* **11**, 473–481
- 15 Villaschi, S. and Spagnoli, L. G. (1983) Autoradiographic and ultrastructural studies on the human fibro-atheromatous plaque. *Atherosclerosis* **48**, 95–100
- 16 Gordon, D., Reidy, M. A., Benditt, E. P. and Schwartz, S. M. (1990) Cell proliferation in human coronary arteries. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4600–4604
- 17 Rosenfeld, M. E. and Ross, R. (1990) Macrophage and smooth muscle cell proliferation in atherosclerotic lesions of WHHL and comparably hypercholesterolemic fat-fed rabbits. *Arteriosclerosis* **10**, 680–687
- 18 Katsuda, S., Coltrera, M. D., Ross, R. and Gown, A. M. (1993) Human atherosclerosis. IV. Immunocytochemical analysis of cell activation and proliferation in lesions of young adults. *Am. J. Pathol.* **142**, 1787–1793
- 19 Reikhter, M. D. and Gordon, D. (1995) Active proliferation of different cell types, including lymphocytes, in human atherosclerotic plaques. *Am. J. Pathol.* **147**, 668–677
- 20 Wang, J., Wang, S., Lu, Y., Weng, Y. and Gown, A. M. (1994) GM-CSF and M-CSF expression is associated with macrophage proliferation in progressing and regressing rabbit atheromatous lesions. *Exp. Mol. Pathol.* **61**, 109–118
- 21 Hamilton, J. A., Myers, D., Jessup, W., Cochrane, F., Byrne, R., Whitty, G. and Moss, S. (1999) Oxidized LDL can induce macrophage survival, DNA synthesis, and enhanced proliferative response to CSF-1 and GM-CSF. *Arterioscler. Thromb. Vasc. Biol.* **19**, 98–105
- 22 Hamilton, J. A., Jessup, W. and Whitty, G. (2000) Oxidized LDL can promote human monocyte survival. *Arterioscler. Thromb. Vasc. Biol.* **20**, 2329–2331
- 23 Sakai, M., Miyazaki, A., Hakamata, H., Sasaki, T., Yui, S., Yamazaki, M., Shichiri, M. and Horiuchi, S. (1994) Lysophosphatidylcholine plays an essential role in the mitogenic effect of oxidized low density lipoprotein on murine macrophages. *J. Biol. Chem.* **269**, 31430–31435
- 24 Matsumura, T., Sakai, M., Kobori, S., Biwa, T., Takemura, T., Matsuda, H., Hakamata, H., Horiuchi, S. and Shichiri, M. (1997) Two intracellular signaling pathways for activation of protein kinase C are involved in oxidized low-density lipoprotein-induced macrophage growth. *Arterioscler. Thromb. Vasc. Biol.* **17**, 3013–3022
- 25 Martens, J. S., Loughheed, M., Gomez-Munoz, A. and Steinbrecher, U. P. (1999) A modification of apolipoprotein B accounts for most of the induction of macrophage growth by oxidized low density lipoprotein. *J. Biol. Chem.* **274**, 10903–10910
- 26 Tushinski, R. J. and Stanley, E. R. (1985) The regulation of mononuclear phagocyte entry into S phase by the colony stimulating factor CSF-1. *J. Cell Physiol.* **122**, 221–228
- 27 Kritharides, L., Jessup, W., Mander, E. L. and Dean, R. T. (1995) Apolipoprotein A-I-mediated efflux of sterols from oxidized LDL-loaded macrophages. *Arterioscler. Thromb. Vasc. Biol.* **15**, 276–289
- 28 Hazell, L. J. and Stocker, R. (1993) Oxidation of low-density lipoprotein with hypochlorite causes transformation of the lipoprotein into a high-uptake form for macrophages. *Biochem. J.* **290**, 165–172
- 29 Gelissen, I. C., Brown, A. J., Mander, E. L., Kritharides, L., Dean, R. T. and Jessup, W. (1996) Sterol efflux is impaired from macrophage foam cells selectively enriched with 7-ketocholesterol. *J. Biol. Chem.* **271**, 17852–17860
- 30 Kritharides, L., Upston, J., Jessup, W. and Dean, R. T. (1998) Accumulation and metabolism of low density lipoprotein-derived cholesteryl linoleate hydroperoxide and hydroxide by macrophages. *J. Lipid Res.* **39**, 2394–2405
- 31 Khoo, J. C., Miller, E., McLoughlin, P. and Steinberg, D. (1988) Enhanced macrophage uptake of low density lipoprotein after self-aggregation. *Arteriosclerosis* **8**, 348–358
- 32 Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) A simple method for the isolation and purification of total lipid from animal tissues. *J. Biol. Chem.* **226**, 497–509
- 33 Kritharides, L., Jessup, W., Gifford, J. and Dean, R. T. (1993) A method for defining the stages of low-density lipoprotein oxidation by the separation of cholesterol- and cholesteryl ester-oxidation products using HPLC. *Anal. Biochem.* **213**, 79–89
- 34 Jaworowski, A., Wilson, N. J., Christy, E., Byrne, R. and Hamilton, J. A. (1999) Roles of the mitogen-activated protein kinase family in macrophage responses to colony stimulating factor-1 addition and withdrawal. *J. Biol. Chem.* **274**, 15127–15133
- 35 Jessup, W., Mohr, D., Gieseg, S. P., Dean, R. T. and Stocker, R. (1992) The participation of nitric oxide in cell free- and its restriction of macrophage-mediated oxidation of low-density lipoprotein. *Biochim. Biophys. Acta* **1180**, 73–82
- 36 Loughheed, M., Loughheed, M., Moore, E., Scriven, D. and Steinbrecher, U. (1999) Uptake of oxidized LDL by macrophages differs from that of acetyl LDL and leads to expansion of an acidic endolysosomal compartment. *Arterioscler. Thromb. Vasc. Biol.* **19**, 1881–1890
- 37 Clare, K., Hardwick, S., Carpenter, K., Weerantunge, N. and Mitchinson, M. (1995) Toxicity of oxysterols to human monocyte-macrophages. *Atherosclerosis* **118**, 67–75
- 38 Bartocci, A., Pollard, J. W. and Stanley, E. R. (1986) Regulation of colony-stimulating factor 1 during pregnancy. *J. Exp. Med.* **164**, 956–961
- 39 Bird, D. A., Gillette, K. L., Horkko, S., Friedman, P., Dennis, E. A., Witztum, J. L. and Steinberg, D. (1999) Receptors for oxidized low-density lipoprotein on elicited mouse peritoneal macrophages can recognize both the modified lipid moieties and the modified protein moieties: implications with respect to macrophage recognition of apoptotic cells. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6347–6352
- 40 Terpstra, V., Bird, D. A. and Steinberg, D. (1998) Evidence that the lipid moiety of oxidized low density lipoprotein plays a role in its interaction with macrophage receptors. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1806–1811
- 41 van Reyk, D. M. and Jessup, W. (1999) The macrophage in atherosclerosis: modulation of cell function by sterols. *J. Leukocyte Biol.* **66**, 557–561
- 42 Tertov, V. V., Sobenin, I. A., Gabbasov, Z. A., Popov, E. G. and Orekhov, A. N. (1989) Lipoprotein aggregation as an essential condition of intracellular lipid accumulation caused by modified low density lipoproteins. *Biochem. Biophys. Res. Commun.* **163**, 489–494
- 43 Hoff, H. F., Whitaker, T. E. and O'Neil, J. (1992) Oxidation of low density lipoprotein leads to particle aggregation and altered macrophage recognition. *J. Biol. Chem.* **267**, 602–609
- 44 Yui, S., Sasaki, T., Miyazaki, A., Horiuchi, S. and Yamazaki, M. (1993) Induction of murine macrophage growth by modified LDLs. *Arterioscler. Thromb. Vasc. Biol.* **13**, 331–337
- 45 Steinberg, D. (1997) Low density lipoprotein oxidation and its pathobiological significance. *J. Biol. Chem.* **272**, 20963–20966
- 46 Sparrow, C. P., Parthasarathy, S. and Steinberg, D. (1989) A macrophage receptor that recognizes oxidized low density lipoprotein but not acetylated low density lipoprotein. *J. Biol. Chem.* **264**, 2599–2604
- 47 Arai, H., Kita, T., Yokode, M., Narumiya, S. and Kawai, C. (1989) Multiple receptors for modified low density lipoproteins in mouse peritoneal macrophages: different uptake mechanisms for acetylated and oxidized low density lipoproteins. *Biochem. Biophys. Res. Commun.* **159**, 1375–1382

Received 31 October 2000/18 December 2000; accepted 24 January 2001