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Minimally Modified LDL Is an Oxidized LDL Enriched with Oxidized Phosphatidylcholines

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The oxidative modification of low-density lipoprotein (LDL) is involved in atherogenesis. Among a variety of modified LDLs mentioned in the literature, so-called minimally modified LDL (MM-LDL) was reported to have pro-atherogenic properties despite minimal changes in its oxidative measures. After treatment of LDL with 1 μM $FeSO_4$ at 4°C for 96 h, the resulting MM-LDL showed a slight increase in thiobarbituric acid-reactive substances (TBARS) and little association with macrophages. On the other hand, heavily oxidized LDL, which was prepared by copper-induced oxidation of LDL at 37°C, showed a sharp increase in TBARS and strong association with macrophages. By introducing a fluorometric procedure to detect aldehyde-containing phosphatidylcholines (aldehyde-PCs), we examined the amounts of aldehyde-PCs in modified LDL preparations. Aldehyde-PCs increased to 23.4 pmol/μg protein in MM-LDL, which was more than four-fold higher than in the heavily oxidized LDL. We conclude that MM-LDL is a unique type of oxidized LDL enriched with aldehyde-PCs.

Key words: oxidized LDL, minimally modified LDL, oxidized phosphatidylcholine, HPLC.

Abbreviations: aldehyde-PCs, aldehyde-containing phosphatidylcholines; apoB, apolipoprotein B; 5CHO-PC, 2-oxovaleroyl phosphatidylcholine; 9CHO-PC, 2-oxononanoyl phosphatidylcholine; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylcarboindocyanine; DPPP, diphenyl-1-pyrenylphosphine; LDL, low-density lipoprotein; MM-LDL, minimally modified low-density lipoprotein; OxLDL, oxidized low-density lipoprotein; OxPC, oxidized phosphatidylcholine; PC, phosphatidylcholine; PPAR, peroxisome proliferator–activating receptor; TBARS, thiobarbituric acid-reactive substances.

The presence of oxidized LDL (OxLDL), which is believed to be involved in atherogenesis, has been proven in atherosclerotic lesions (*1*, *2*) and circulating plasma (*3–5*), using specific monoclonal antibodies. OxLDL induces various responses of macrophages including foam cell formation and proliferation (*6*, *7*). OxLDL also shows stimulatory effects on endothelial cells and smooth muscle cells (*8–10*).

Of the variety of experimental conditions used to prepare OxLDL, $CuSO_4$ is the most widely used oxidant in OxLDL preparation (*11*). Copper ions catalyze the cleavage of lipid peroxides and initiate radical chain reactions of lipid peroxidation in LDL. Aldehyde-containing products generated during LDL oxidation, including aldehyde-containing phosphatidylcholines (aldehyde-PCs), modify apolipoprotein B (apoB) by binding to amino groups (*11*).

Bioactive oxidized phosphatidylcholines (OxPCs) are found in OxLDL. For example, 2-oxovaleroyl PC (5CHO-PC) exhibits platelet-activating factor (PAF)-like properties (*12*), as well as monocyte chemoattractant protein-1 (MCP-1) induction in endothelial cells (*13*). Since the binding and uptake of OxLDL by macrophages are inhibited by either aldehyde-PCs or monoclonal antibodies recognizing 2-oxononanoyl PC (9CHO-PC) and 5CHO-PC, the aldehyde-PCs are postulated to form OxPC-apoB adducts that are involved in OxLDL recognition by macrophages (*14*, *15*). Aldehyde-PCs are also reported to be putative ligands for peroxisome proliferator–activating receptor (PPAR) α (*16*). Thus, aldehyde-PCs could be good markers for oxidative modification of LDL.

Minimally modified LDL (MM-LDL) has been reported to exhibit strong stimulatory activity toward endothelial cells in culture (*17*). MM-LDL is thought to be pro-atherogenic since it has a variety of pro-inflammatory functions including induction of MCP-1 (*17–19*). MM-LDL has been reported to show only a moderate increase in the TBARS value and is not recognized by scavenger receptors, although oxidative modification is likely to be involved (*17*). To understand possible mechanisms of atherogenesis, it is necessary to elucidate the biochemical background of MM-LDL by comparison with copper-induced OxLDL. By introducing a fluorometric HPLC procedure to detect aldehyde-PCs, we found that there is extensive accumulation of aldehyde-PCs in MM-LDL despite its low TBARS value. We conclude that MM-LDL is a type of oxidatively-modified LDL that is enriched with aldehyde-PCs.

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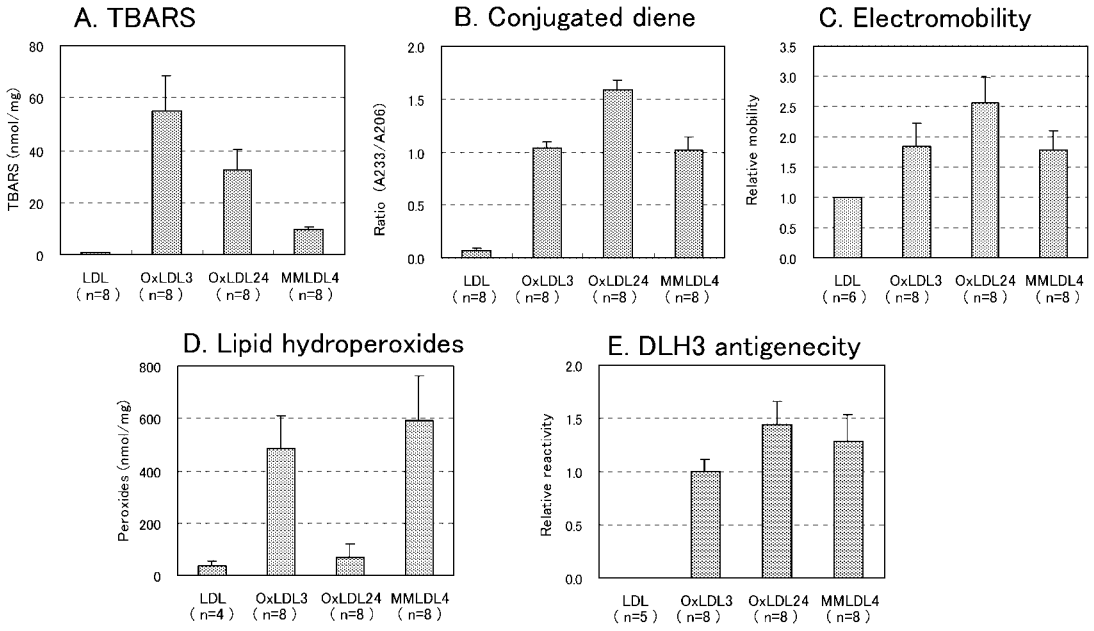

Fig. 1. **Oxidation parameters for copper-induced OxLDL and iron-induced MM-LDL.** $OxLDL_3$ was prepared by incubating LDL (0.2 mg/ml) with 5 μM $CuSO_4$ at 37°C for 3 h, while $OxLDL_{24}$ was prepared by incubating LDL (2 mg/ml) with 50 μM $CuSO_4$ at 37°C for 24 h. MM-LDL was prepared by dialyzing LDL (0.2 mg/ml) against PBS containing 1 μM $FeSO_4$ at 4°C for 96 h. For each sample, the TBARS value (A), UV absorbance corresponding to conjugated dienes (B), electromobility on agarose gel electrophoresis (C), hydroperoxides in a total lipid extract (D), and oxidized PC modification of LDL particles, as judged by means of a sandwich ELISA method using anti-oxidized PC antibody DLH3 (E), were determined. In each figure, error bars indicate standard deviations.

EXPERIMENTAL PROCEDURES

Materials—An anti-OxPC monoclonal antibody, DLH3, was prepared as described previously (*2*). Cyclohexanedione was purchased from Nacalai Tesque, Kyoto. Diphenyl-1-pyrenylphosphine (DPPP) was purchased from Wako Pure Chemical, Osaka. DiI(C3) was purchased from Molecular Probe. Murine macrophage cell line J774.1 was from the American Type Culture Collection. Bovine lipoprotein-deficient serum was obtained from Sigma.

Preparation of OxLDL and MM-LDL—LDL was prepared from human plasma by stepwise density gradient centrifugation (*3*). OxLDL was prepared under two different sets of experimental conditions: $OxLDL_3$ was prepared by incubating LDL (0.2 mg/ml) with 5 μM $CuSO_4$ in phosphate-buffered saline (PBS) at 37°C for 3 h. The reaction was stopped by adding EDTA (final, 0.25 mM). These are the general experimental conditions we have been using for the preparation of OxLDL (*2–4*), which are also used by other groups (*20*). $OxLDL_{24}$ was prepared by incubating LDL (2.0 mg/ml) with 50 μM $CuSO_4$ at 37°C for 24 h (*21*). Ferrous ion-induced MM-LDL was prepared according the methods used by Berliner *et al*. (*17*, *18*). Briefly, LDL (0.2 mg/ml) was dialyzed against PBS containing 1 μM $FeSO_4$ at 4°C for 96 h.

Measurement of aldehyde-PCs—The amounts of aldehyde-PCs were determined as fluorescent derivatives by HPLC. The procedure used is a modification of a method for measuring aldehyde-containing cholesteryl esters (*22*). Standard aldehyde-PCs (9CHO-PC and 5CHO-PC) were prepared from 1-palmitoyl-2-oleoyl PC and 1-stearoyl-2-arachidonoyl PC, respectively, using osmium tetroxide (*23*). Total lipids, extracted from 80 μg of OxLDL or MM-LDL using chloroform and methanol (*24*), were dissolved in 160 μl of chloroform:methanol (2:1) in siliconized tight-sealed glass tubes. Following the addition of 16 μl of reagent (1:1 mixture of 1,3-cyclohexanedione (200 mg/ml in ethanol) and ammonium acetate (600 mg/ml in 20% acetic acid) solutions) the reaction mixture was heated at 75°C for 60 min. After the reaction mixture has been re-dissolved in 80 μl of methanol:acetonitrile (7:3), 10 μl of the resultant solution was injected into the HPLC apparatus. HPLC analysis was performed with the following parameters: LiChrosorb RP18 column (4 × 250 mm, Merck), a gradient elution with methanol:acetonitrile:water containing 20 mM choline chloride with an initial solvent ratio of 651:279:70 and a final ratio of 672:288:40 within 30 min, and a flow rate of 1 ml/min. Fluorescent intensity was monitored using a fluorescence detector with excitation at 366 nm and emission at 455 nm.

A lipid extract treated with cyclohexanedione or fractions recovered on HPLC were analyzed on a TLC plate (Silica gel 60; Merck 5748) with a solvent system of chloroform/methanol/water = 130:360:11. Fluorescent derivatives were detected under UV light using a LumiVision Pro photoimaging system (Aisain Seiki, Kariya). Phospholipids were detected by spraying Dittmer reagent (*25*).

Association of OxLDL with Macrophages—Lipoproteins were labelled with fluorescent probe DiI(3) as described in (*26*). J774 macrophages (1×10^5 cells/ml) were placed in slide chamber wells and then incubated with serum-free RPMI1640 medium containing 10 μg/ml LPS for 16 h. After washing with RPMI1640, the cells were incubated with RPMI1640 containing 0.2% lipoprotein-defi-

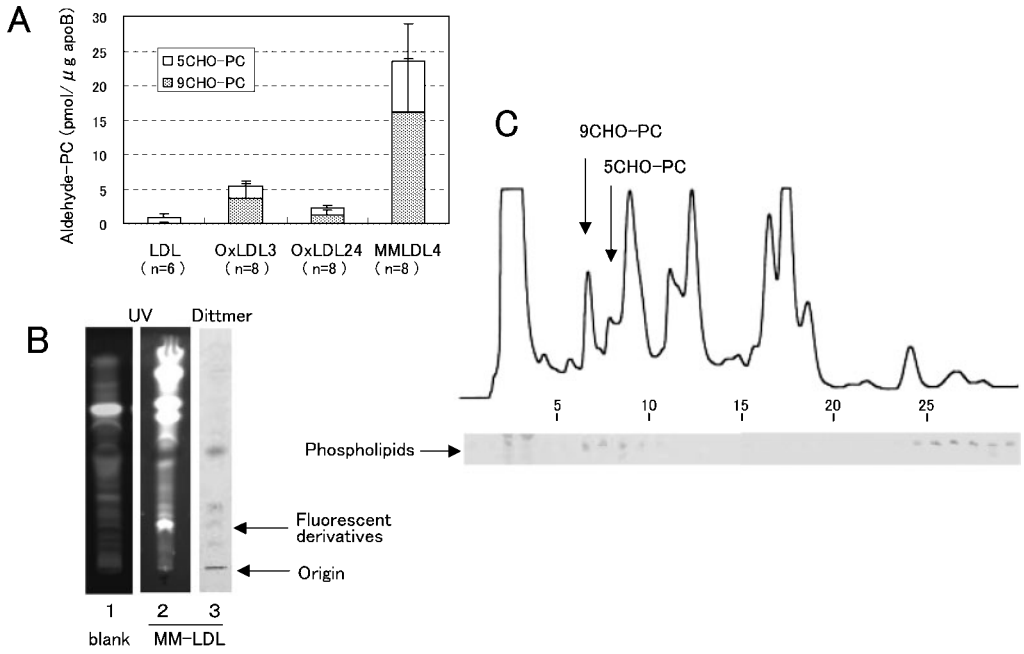

Fig. 2. **Accumulation of aldehyde-PCs in MM-LDL.** (A) $OxLDL_3$, $OxLDL_{24}$, and MM-LDL were prepared as in Fig. 1. Total lipids extracted from these lipoproteins were treated with cyclohexanedione to obtain fluorescent derivatives of aldehyde-containing products. Aldehyde-PCs were deteremined by HPLC as described under Experimental Procedures. Error bars indicate standard deviations. (B) TLC profile of the total lipid extract of MM-LDL after cyclohexanedione treatment. The sample was developed with chloroform/methanol/water = 130:60:11, then fluorescent derivatives were visualized under UV light, and phospholipids were detected as blue spots by spraying Dittmer reagent. An excess amount of cyclohexanedione appears as a yellow band. (C) HPLC profile of the total lipid extract of MM-LDL after cyclohexanedione treatment. The peaks corresponding to fluorescent derivatives derived from standard 9CHO-PC and 5CHO-PC are indicated by arrows. Eluates collected every minute were subjected to TLC and then sprayed with Dittmer reagent to detect phospholipids as blue spots.

cient serum and 0.1 μg/ml DiI-labelled lipoproteins at 37°C for 5 h. To determine competitive effects, 25 μg/ml unlabelled lipoproteins were added together with the DiI-OxLDL. The cells were washed with PBS and then fixed with 4% formalin-PBS.

Other Methods—A sandwich ELISA procedure was carried out using anti-OxPC monoclonal antibody DLH3 and an anti-human apoB polyclonal antibody (*3*). TBARS values were measured by the method of Beuge and Aust (*27*). The formation of conjugated diene was quantitated by measuring the absorbance at 233 nm and 206 nm for total lipids extracted from modified lipoproteins (in an ethanol solution). The amounts of lipid hydroperoxides were determined fluorometrically using DPPP as described by Akasaka *et al*. (*28*).

RESULTS

Preparation of OxLDL and MM-LDL—LDL was treated with either copper or ferrous ions under different conditions. Following incubation of 0.2 mg/ml LDL with 5 μM $CuSO_4$ at 37°C for 3 h, OxLDL with a high TBARS value (55.0 nmol/mg LDL) was obtained; this preparation is referred to as $OxLDL_3$. This is one of the typical procedures used to prepare OxLDL, as described in the literature (*2–4*, *9*). Incubation of LDL (2.0 mg/ml) with 50 μM $CuSO_4$ at 37°C for 24 h ($OxLDL_{24}$) gave a slightly decreased, but still significant, TBARS value (Fig. 1A). MM-LDL prepared by dialyzing LDL (0.2 mg/ml) against PBS containing 1 μM $FeSO_4$ at 4°C for 96 h showed only a slight increase in the TBARS value, which corresponds to values reported in the original paper (*17*).

Besides the moderate increases in the TBARS value, another characteristic of MM-LDL is its inability to bind to macrophage scavenger receptors (*17*). When J774.1 macrophage cells were incubated with DiI-$OxLDL_{24}$ for 5 h, strong cellular association of DiI-OxLDL was observed. This cellular association was abolished by adding an excess amount of cold $OxLDL_{24}$ but not by MM-LDL or native LDL (data not shown). Similarly, the cellular association of DiI-$OxLDL_3$ was not blocked by an excess amount of unlabelled MM-LDL or native LDL. When MM-LDL or native LDL labelled with DiI was used as the probe, no significant cellular association was observed (data not shown). These results suggest that our MM-LDL preparations does not share macrophage scavenger receptors with OxLDL.

MM-LDL Is Oxidatively Modified—To characterize the modified nature of these lipoproteins, we examined other markers for oxidative modification. Conjugated dienes as well as lipid hydroperoxides are likely to be generated in the early phase of lipid peroxidation, although TBARS values are related to secondary products generated through cleavage of acyl chains. Modification of apoB by lipid peroxidation products would endow the particles with net negative charges, which would thereby increase the electromobility in an agarose gel. A sandwich ELISA using anti-OxPC antibody DLH3 and an anti-apoB antibody

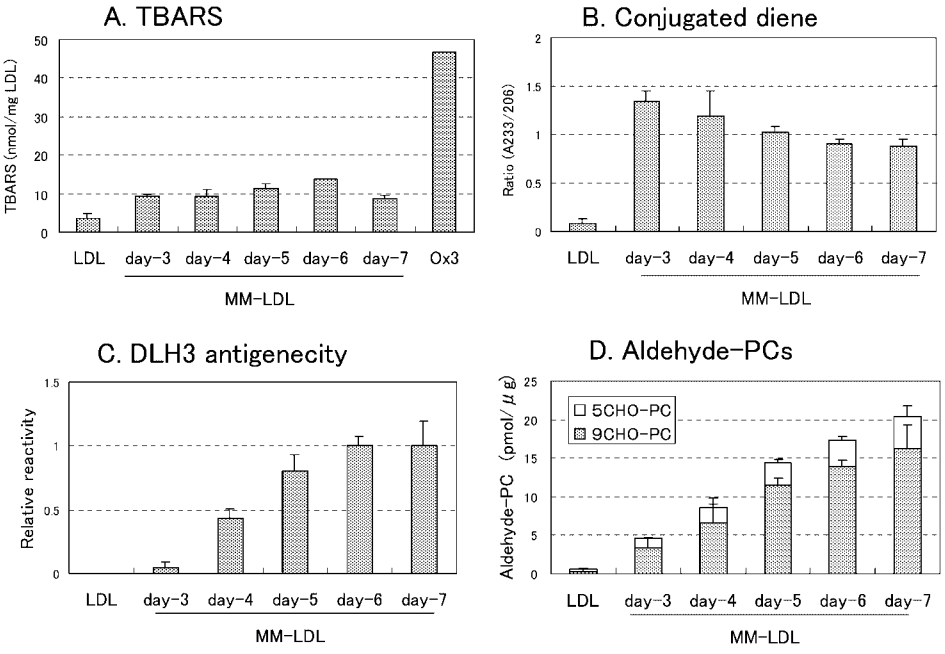

Fig. 3. **Time course of oxidative modification of MM-LDL.** LDL (0.2 mg/ml) was dialyzed against PBS containing 1 μM $FeSO_4$ at 4°C for 3 to 7. For each sample, TBARS (A), conjugated dienes (B), lipid hydroperoxides (D), and reactivity to anti-oxidized PC antibody DLH3 (E) were determined. In each figure, error bars indicate standard deviations for triplicate preparations.

detects the formation of apoB-OxPC adducts (*3*, *14*). We found that all of these oxidative markers for MM-LDL increased to much the same extent as $OxLDL_3$, indicating that MM-LDL is already oxidatively modified (Fig. 1, B–E).

MM-LDL Is Enriched with Aldehyde-PCs—We introduced a fluorometric assay for two aldehyde-PCs, 9CHO-PC and 5CHO-PC. Treatment of aldehyde compounds with cyclohexanedione and ammonium ions generates a decahydroacridine-1,8-dione structure, a fluorescent chromophore (*22*). The fluorescent derivatives derived from 9CHO-PC and 5CHO-PC were detected by reversed phase HPLC, and it was found that the accumulation of aldehyde-PCs in MM-LDL was four-fold greater than that in $OxLDL_3$ (Fig. 2A). $OxLDL_{24}$ contained much less aldehyde-PCs as well as lipid hydroperoxides, suggesting that these oxidized lipids could be converted into other secondary products (Figs. 2A and 1D).

When cyclohexanedione-treated MM-LDL lipids were analyzed by TLC and reversed-phase HPLC, as shown in Fig. 2B, a band of fluorescent derivative(s) corresponding to a phosphorous-positive blue band was observed on TLC. On reversed-phase HPLC, the peaks of fluorescent derivatives derived from 9CHO-PC and 5CHO-PC appeared at 7 min and 8.5 min, respectively, as compared with standard aldehyde-PCs (Fig. 2C). Then the eluate was fractionated every minute to detect the presence of phospholipids on TLC, phospholipids being eluted at 7–9 min, which corresponds to the retention times of aldehyde-PCs (Fig. 2C, lower panel). Phosphorous-positive bands were also observed at 24–30 min, and faint bands were detected at 3–4 min. Judging from the results of separate experiments, lysoPC was eluted at 3–4 min and PC hydroperoxides around 27 min, and unoxidized diacyl PC needs more than 30 min to be eluted from the column (data not shown). These data suggest that 9CHO-PC and 5CHO-PC are major components of the aldehyde-PCs formed, and that the most of the unidentified fluorescent peaks in the chromatogram are not oxidized PCs.

Fe-Induced Oxidation of LDL Is a Slow Process—LDL was dialyzed against PBS contianing 1 μM $FeSO_4$ at 4°C for up to 7 d (Fig. 3). Significant conjugated diene has been formed by day 3 and then decreased gradually, suggesting that the lipid peroxidation reaction had already proceeded. TBARS showed a mild increase throughout the incubation period, the amount being less than that of copper-induced $OxLDL_3$. On the other hand, the reactivity to the DLH3 antibody and the amount of aldehyde-PCs were minimal on day 3, and then increased gradually during dialysis for up to 7 d.

Effects of Dialysis and Low Temperature—There are three major differences in the experimental conditions for MM-LDL and OxLDL preparation. Firstly, a low concentration of ferrous ions (1 μM) is used instead of copper ions. It has been shown that copper ions oxidize LDL more efficiently than ferrous ions (*29*). Secondly, the reaction is performed at 4°C instead of 37°C. Thirdly, MM-LDL is incubated in a dialysis bag with a large volume of external buffer containing ferrous ions, while OxLDL is incubated in a test tube, in which a small volume of a copper solution is mixed directly with LDL. To determine the possible effects of dialysis on the LDL modifications, we prepared two other types of modified LDL: LDL was either dialyzed against PBS containing 5 μM $CuSO_4$ at 37°C for 3 h (Cu-LDL-dialysis), or was mixed with 1 μM $FeSO_4$ and then incubated in a test tube at 4°C for 96 h (Fe-LDL-tube). These two preparations differ from $OxLDL_3$ and MM-LDL only in terms of the reaction container, namely, a dialysis bag or a test tube. Cu-LDL-dialysis gave a low TBARS value, relatively high amounts of conjugated dienes and lipid hydroperoxides, and significant antigenicity (Fig. 4, A–D). The accumulation of aldehyde-PCs was 2.6-fold higher than that of $OxLDL_3$ (Fig. 4E). Conversely, oxidative modification in the Fe-LDL-tube was minimal, only intermediate increases in diene formation and lipid hydroperoxides being observed. These results suggest that treatment of LDL in a dialysis

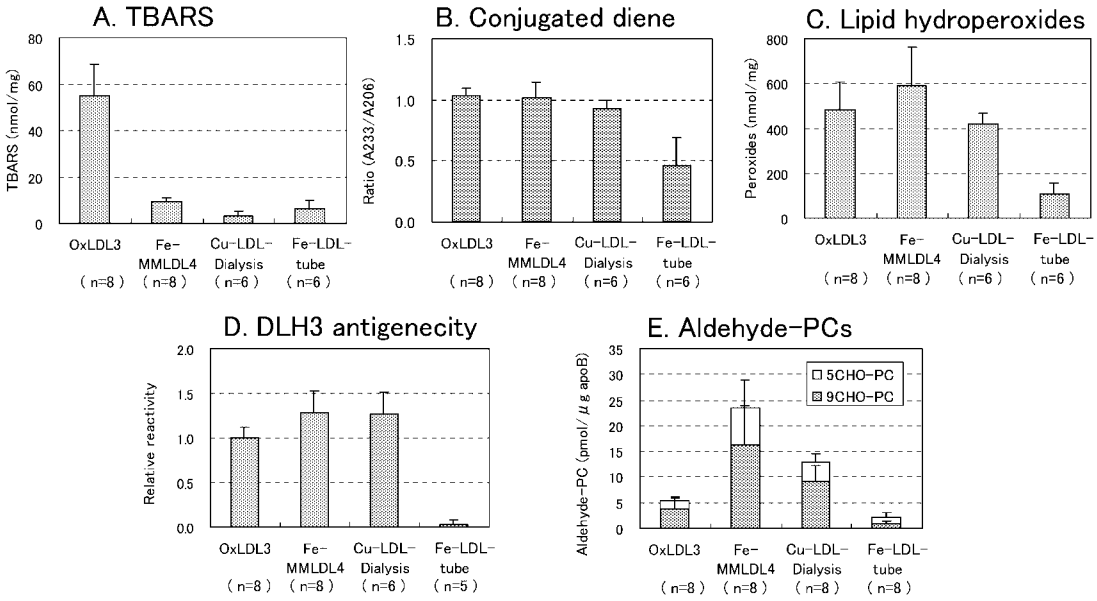

Fig. 4. **Effect of dialysis on the oxidative modification of LDL.** LDL was treated with 5 μM $CuSO_4$ at 37°C for 3 h in either a test tube or a dialysis bag ($OxLDL_3$ and Cu-LDL-dialysis), or alternatively LDL was treated with 1 μM $FeSO_4$ at 4°C for 96 h in either a dialysis bag or a test tube (MM-LDL4 and Fe-LDL-tube). In each figure, error bars indicate standard deviations.

bag during the oxidation reaction contributes to the MM-LDL-type modification, namely the enrichment of OxPCs in spite of a low TBARS value.

DISCUSSION

In the current study, we clearly showed that MM-LDL is a unique type of oxidatively modified LDL that is enriched with aldehyde-PCs. Although mounting evidence strongly suggests the involvement of OxLDL in atherogenesis, the real nature of OxLDL present *in vivo* has yet to be fully clarified. Recently, MM-LDL was reported to show a series of strong pro-atherogenic properties. However, the precise relationship between MM-LDL and OxLDL remains obscure. In this study, by examining several modified LDL preparations side-by-side, we demonstrated for the first time that iron-induced MM-LDL is oxidized and can be characterized by enrichment of aldehyde-PCs.

Attention has been paid to OxPCs as bioactive lipids involved in disease states including atherosclerosis and inflammatory disorders. An aldehyde-PC, 5CHO-PC (also referred to as POVPC in the literature), has been shown to induce endothelial expression of MCP-1 (*13*) and VCAM-1 (*30*), as well as activation of PPARα in endothelial cells (*16*). It also activates human neutrophils to cause cellular adhesion to a gelatin-coated matrix at a low concentration (*12*). Watson *et al*. beautifully demonstrated that OxPCs present in MM-LDL are responsible for some of the biological properties of endothelial activation (*13*). According to them, as low as 1 nmol/ml of 5CHO-PC induces endothelial activation. Our present data, showing that MM-LDL is enriched with aldehyde-PCs, where more than 2 nmol of aldehyde-PCs are formed in 100 μg of MM-LDL, strongly support the previous observation that MM-LDL is a potent bioactive modified LDL in vascular cells, although aldehyde-PCs must be a part of the bioactive products in oxidatively modified lipoproteins.

Our data showed that MM-LDL accumulated a greater amount of aldehyde-PCs than $OxLDL_3$ or $OxLDL_{24}$ (Fig. 2A), although they all exhibited comparative reactivity to an anti-OxPC antibody, DLH3, on sandwich ELISA (Fig. 1E). It is important to note that aldehyde-PCs can either associate with LDL particles as free phospholipid molecules or bind to LDL covalently as adducts on the apoB protein. The aldehyde-PCs determined by HPLC in this study are extractable products, namely, they are free aldehyde-PCs. The DLH3 antibody, however, does not distinguish the free aldehyde-PCs from their adducts with proteins (*3*, *31*). It is likely that MM-LDL is relatively rich in free aldehyde-PCs, and the adducts of aldehyde-PCs on the apoB protein may be enriched in OxLDLs.

MM-LDL was antigenic to DLH3 antibody (Fig. 1E), however, MM-LDL showed little association with macrophages (data not shown). It is likely that scavenger receptors can recognize multiple epitopes generated on OxLDL, while DLH3-positive OxPC-apoB adducts may only comprise a part of them. Recently, Hazen and his colleagues identified a series of oxidized PCs as CD36 ligands (*32*). They reported that oxidized PC molecules containing a terminal γ-oxo-α,β-unsaturated carbonyl (aldehyde or carboxylic acid) structure exhibited higher affinity to CD36 than aldehyde-PCs. The amounts of 5CHO-PC, however, found in copper-induced OxLDL and atherosclerotic lesions in WHHL rabbits are higher than those of the oxidized PCs containing the γ-oxo-α,β-unsaturated carbonyl structure (*33*). It will be important to elucidate the whole composition of the variety of oxidized lipids in modified lipoproteins in the future.

The TBARS value for $OxLDL_{24}$ was lower than that for $OxLDL_3$. Also, aldehyde-PCs are likely to be reduced during prolonged incubation, suggesting that the oxidation

products formed in the early phase could be reduced through secondary decomposition, condensation, or formation of adducts with apoB (*21*, *31*, *34*). Similarly, the enrichment of aldehyde-PCs in MM-LDL might be explained by inefficient secondary decomposition. When the concentrations of TBARS were kept at low levels by dialysis during the reaction period, consumption of aldehyde-PCs after reaction with water soluble aldehydes would be reduced, and hence aldehyde-PCs could accumulate in the modified lipoproteins.

Despite the low TBARS values, our MM-LDL preparations showed good increases in diene formation, lipid hydroperoxides, antigenicity to the DLH3 antibody and aldehyde-PCs, indicating that the MM-LDL is actually oxidized. The major components of TBARS are soluble small molecules, whereas diene-containing long acyl chains, OxPC and epitopes generated in OxLDL are associated with lipoprotein particles. Treatment of LDL in dialysis bags would allow low molecular and water-soluble TBARS to escape easily through the dialysis membranes. The TBARS value for the copper-induced OxLDL prepared in a dialysis bag at 37°C (OxLDL-dialysis) was low (Fig. 4).

When LDL was treated with ferrous ions in a test tube at 4°C, the oxidative change was even milder than that for MM-LDL prepared by dialysis. Although the reason for this result has yet to be determined, one possibility is that total amount of ferrous ions in the reaction system rather than their concentration may be another factor for the lipid peroxidation reaction.

In vessel walls, the physiological location of LDL modification, tissue fluid surrounding the LDL particles is likely to circulate continuously, and it can be speculated that MM-LDL prepared using dialysis bags could represent oxidatively-modified LDL present *in vivo*. Many research groups have developed a variety of experimental conditions for preparing OxLDL and MM-LDL; it would thus be informative to determine the amounts of OxPCs accumulated in modified lipoproteins, since the amounts of OxPCs in these preparations might be crucial contributors to the biological and pathological responses.

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