Oxidized Phosphatidylcholines That Modify Proteins

ANALYSIS BY MONOCLONAL ANTIBODY AGAINST OXIDIZED LOW DENSITY LIPOPROTEIN*

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Oxidatively modified low density lipoprotein (OxLDL) is known to be involved in atherogenesis. We have previously developed a murine monoclonal antibody, FOH1a/DLH3, which recognized oxidatively modified lipoproteins as well as foam cells in human atherosclerotic lesions (Itabe, H., Takeshima, E., Iwasaki, H., Kimura, J., Yoshida, Y., Imanaka, T., and Takano, T. (1994) J. Biol. Chem. 269, 15274–15279). The antigen of this monoclonal antibody was formed by peroxidation of phosphatidylcholine (PC), and the antigenic oxidized PC (OxPC) derivatives are thought to form complexes with polypeptides including apolipoproteins. OxLDL was measured by a sensitive sandwich enzyme-linked immunosorbent assay using the monoclonal antibody and anti-human apolipoprotein B antibody, in which antigenic OxPC competed with OxLDL. When antigenic activities of PC analogs were tested by the competition assay, 1-palmitoyl-2-(9-oxononanoyl) PC (9-CHO PC) and the hydroperoxide of egg PC potently inhibited the detection of OxLDL. 1-Palmitoyl-2-linoleoyl PC was oxidized with ferrous ion and ascorbic acid, and the antigenic products were purified from the OxPC extracts on high pressure liquid chromatography columns and subsequently analyzed by laser desorption mass spectrometry. Molecular weight determination and retention times of high pressure liquid chromatography suggest that one of these products was 9-CHO PC. Other products are thought to be 8-carbon aldehyde, dihydroxy, and ketohydroxy derivatives of PC. When a C-terminal 16-mer synthetic peptide of the 70-kDa peroxisomal membrane protein was simply incubated with 9-CHO PC, it was found to be reactive in a sandwich enzyme-linked immunosorbent assay using FOH1a/DLH3 and an anti-peptide antiserum. These results suggest that the anti-OxLDL monoclonal antibody FOH1a/DLH3 reacts with several oxidized products of PC including aldehyde derivatives of PC, which covalently modify polypeptides.

Atherosclerosis is a complex vascular disorder that can lead to more serious conditions such as myocardial infarction. Many biological factors are involved in the generation and progression of atherosclerosis, but a major factor in the early stages of atherosclerosis is known to be oxidatively modified low density lipoprotein (OxLDL), which promotes foam cell formation (1). The formation of lipid-laden foam cells from macrophages has been demonstrated by incubation of OxLDL with macrophages in vitro (2, 3), although macrophages did not accumulate lipid droplets when incubated with native LDL. Although OxLDL provides excess amounts of lipids to macrophages, it may also affect atherogenesis due to induction of various cellular responses (4–13). Such responses include induction of endothelin secretion from endothelial cells and macrophages by OxLDL (5, 6), expression of platelet-derived growth factor mRNA in smooth muscle cells (8), and activation of a C-protein-mediated signal transduction pathway (9, 10). Minimally modified LDL, which is a very mildly oxidized form of LDL, induced the expression of monocyte chemotactic protein-1 (11, 12) and tissue factors in endothelial cells (13). However, the active components in OxLDL and the mechanism by which these cellular responses are induced are not yet understood.

Upon oxidation of LDL, apolipoprotein B (apoB) is modified by a wide variety of oxidative products of LDL lipids. Malondialdehyde (MDA) and 4-hydroxynonenal, well known aldehyde-containing products of lipid peroxidation, were detected in OxLDL and atherosclerotic lesions (14–16). Increased mobility on agarose gel electrophoresis as well as a marked reduction in lysine residues suggested that these peroxidation products modified the lysine residues (17, 18). Aggregation of LDL as well as fragmentation of apoB have been demonstrated (18). However, little has been clarified on the structure of OxLDL, partly due to the technical difficulties encountered when analyzing heterogeneous complexes of lipids and the huge apoB protein.

We have shown in previous studies that apoB is modified by oxidized products of phosphatidylcholine (PC) as demonstrated by an anti-OxLDL monoclonal antibody, FOH1a/DLH3 (19). This antibody was produced by immunizing against homoge-

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1 The abbreviations used are: OxLDL, oxidized LDL; LDL, low density lipoprotein; apoB, apolipoprotein B; BSA, bovine serum albumin; MDA, malondialdehyde; PC, phosphatidylcholine; OxPC, oxidized phosphatidylcholine; 5-CHO PC, 1-palmitoyl-2-(5-oxovaleryl) phosphatidylcholine; 8-CHO PC, 1-palmitoyl-2-(8-oxononanoyl) phosphatidylcholine; 9-CHO PC, 1-palmitoyl-2-(9-oxononanoyl) phosphatidylcholine; 5-CHOOH PC, 1-palmitoyl-2-glutaroyl phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide(s); FMP70-C, 70-kDa peroxisomal membrane protein C-terminal synthetic peptide; TLC, thin layer chromatography; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; RP, reverse phase; PBS, phosphate-buffered saline (pH 7.4); PAF, platelet activating factor.
nates of human atheroma and selecting the hybridomas by reactivity to copper-induced OxLDL. The antibody recognizes oxidized products of PC in OxLDL and does not react to MDA-treated LDL, acetylated LDL, or native LDL. Moreover, immunochemical studies showed a colocalization of the antigenic materials of this antibody with macrophage-derived foam cells in human atherosclerotic lesions. In the present study, we isolated and characterized the antigenic OxPC products in order to investigate the molecular basis of oxidative modification of LDL. We found that this antibody reacted to several OxPC products including aldehyde derivatives of PC that modify polypeptides.

**EXPERIMENTAL PROCEDURES**

**Materials**—A partially purified murine monoclonal antibody, FOH1a/DLH3, was prepared from murine ascites by ammonium sulfate precipitation followed by gel filtration on a Toyopearl HW-65® column (Tosoh, Tokyo, Japan) as described previously (20). Sheep anti-human apoB antibody (IgG fraction) was purchased from Binding Site Inc. (Birmingham, United Kingdom). Alkaline phosphatase-conjugated key anti-sheep IgG antibody, which is preabsorbed with human, rat, bovine, and murine sera, was from Chemicon Co. (Temecula, LA). 1-Palmitoyl-2-linoleoyl PC was purchased from Avanti Co. (Perham, AL). 2-Oleoyl lyso-PC was purchased from Sigma. Aldehyde-containing PC analogs, 1-palmitoyl-2-(5-oxovaleroyl) PC (5-CHO PC) and 1-palmitoyl-2-glutaroyl PC were prepared by exchange of 1-palmitoyl-2-oleoyl PC and 1-palmitoyl-2-arachidonoyl PC, respectively (21). 1-Palmitoyl-2-gluutaraldehyde PC was prepared from 1-palmitoyl lyso-PC and glutaric anhydride by the method of Gupta et al. (23). PC hydroperoxides (PCOOH) prepared as described previously (19). Briefly, 1-palmitoyl-2-linoleoyl PC (2 mM), which had been dissolved under argon gas was suspended in phosphate-buffered saline, pH 7.4 (PBS). Ascorbic acid and FeSO4 were dissolved in distilled water just before use. The reaction mixture containing 0.4 mM PC, 0.4 mM ascorbic acid, and 40 μM FeSO4 was incubated at 37°C for 3 h. The reaction was stopped by adding 0.25 mM EDTA.

**Preparation of OxPC and OxPC-Peptide Complex**—OxPC was prepared as described previously (19). Briefly, 1-palmitoyl-2-linoleoyl PC (2 mM), which had been dissolved under argon gas was suspended in phosphate-buffered saline, pH 7.4 (PBS). Ascorbic acid and FeSO4 were dissolved in distilled water just before use. The reaction mixture containing 0.4 mM PC, 0.4 mM ascorbic acid, and 40 μM FeSO4 was incubated at 37°C for 3 h. The reaction was stopped by adding 0.25 mM EDTA.

**Detection of Antigenic OxPC**—Detection of the antigenic OxPC products was achieved using a competition ELISA assay, which is a modification of a sandwich ELISA procedure for OxLDL determination (20). Briefly, microtiter wells precoated with the monoclonal antibody FOH1a/DLH3 (5 μg/ml in PBS, 100 μl/well) were blocked with 1% bovine serum albumin (BSA) in 50 mM Tris-buffered saline, pH 8.0. To the wells were added 100 μl of samples, which were resuspended in PBS and left at room temperature for 30 min, followed by the addition of 10 μl of OxLDL (1 μg/ml). The remaining OxLDL, left after washing with Tris-buffered saline containing 0.05% Tween 20, was detected by 100 μl of sheep anti-human apoB antibody and 100 μl of alkaline phosphatase-conjugated donkey anti-sheep IgG antibody. The reactivity of alkaline phosphatase was measured by incubating 1 mg/ml of p-nitrophenylphosphate at 37°C for appropriate time intervals. The antigenic activity was expressed as percentage of inhibition, calculated as (Abs(OxLDL) - Abs(sample + OxLDL)) × 100/Abs(OxLDL) - Abs(PBS).

**Purification of the Antigenic OxPC**—OxPC extract was fractionated on a semipreparative silica gel column (Merck, LiChrosorb Si60, 7.5 × 250 mm) eluted isocratically with hexane-2-propanol/water (45/50/8) at 3 ml/min. The elution profile was monitored by absorbance at 206 nm. The major peaks with antigenic activities were recovered in four fractions (fractions I–IV) and dried under reduced pressure. The rotary evaporator was operated after the system was filled with argon gas. These fractions were then further separated on an ODS column (Merck, LiChrosorb RP-18, 4 × 250 mm) eluted with a gradient of methanol/water (88/12) to (98/2). The partially purified fractions were then rechromatographed twice on the same column using another solvent system system, methanol/acetonitrile/water (616/264/120 to 684/296/20).

**Detection of CHO-PC and PCOOH**—Two synthetic PC analogs, 9-CHO-PC and egg PCOOH were subjected to HPLC under the same conditions as were used in the second step of the antigen purification (see above). Eluates, collected at 1-min intervals, were developed by TLC (Polygram Sil G) with chloroform/methanol/water (10/5/1). The major peaks with antigenic activities were recovered in four fractions (fractions I–IV) and dried under reduced pressure. The rotary evaporator was operated after the system was filled with argon gas. These fractions were then further separated on an ODS column (Merck, LiChrosorb RP-18, 4 × 250 mm) eluted with a gradient of methanol/water (88/12) to (98/2). The partially purified fractions were then rechromatographed twice on the same column using another solvent system, methanol/acetonitrile/water (616/264/120 to 684/296/20).

**Separation of the OxPC-Peptide Complex**—Purification of the PMP70-C and separation of the OxPC-peptide complex were carried out on an HPLC column (TSKgel-ODS80TM, 6 × 150 mm, Tosoh Co., Tokyo, Japan) eluted with acetonitrile/water containing 0.1% of trifluoroacetic acid (10/90 to 90/10) at 1.2 ml/min. The absorbance at 220 nm was monitored. Eluates were collected at 1-min intervals, and the OxPC-peptide complex was detected by a sandwich ELISA using FOH1a/DLH3 and the anti-PMP70-C antisera.

**Mass Spectrometry**—Two authentic standards, 1-palmitoyl-2-linoleoyl PC and 9-CHO-PC, and purified antigenic products were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a Vision 2000 (Finnigan MAT, San Jose, CA) equipped with a N2 laser (337 nm, 2-ns pulse duration). The matrix used was 2,5-dihydroxybenzoic acid dissolved in distilled water at a concentration of 10 mg/ml. For matrix-assisted laser desorption/ionization analysis, 1 μl of the sample solution was mixed with an equal volume of the matrix solution on the target. The spectra represent the accumulation of 10 single laser shots. They were calibrated using angiotensin II (1046 Da) as standard spotted on the same target.

**Fig. 1. Inhibition by various PC analogs of OxLDL binding to the monoclonal antibody FOH1a/DLH3.** To the microtiter wells precoated with 0.5 μg of FOH1a/DLH3 were added 0.1 (hatched bar) or 1 nmol (closed bar) of the various PC analogs dispersed in PBS. Then 10 ng of copper-induced OxLDL were added to each well, and a competition assay was carried out as described under “Experimental Procedures.” Values are mean ± S.D. of three experiments expressed as relative reactivity, in which the control with no PC analog was taken as 100%.

**TABLE**

| PC | OxPC | 9-CHO PC | LysoPC | 5-CHO PC | POOH | PCOOH |
|----|------|----------|--------|----------|-------|-------|
| ![Graph](image-url) | ![Graph](image-url) | ![Graph](image-url) | ![Graph](image-url) | ![Graph](image-url) | ![Graph](image-url) | ![Graph](image-url) |
Fig. 2. Elution profiles of the fractions I-IV on a reverse phase HPLC. An aliquot (100 nmol of phosphorus) of 9-CHO PC (A) and egg PCOOH (C) used in Fig. 1 were injected into an ODS column (LiChrosorb RP-18) and eluted with a methanol/water gradient (88/12) to (98/2) as described under "Experimental Procedures." UV detection at 206 nm was carried out (solid line). The antigenic activity in each 1-min fraction was indicated as percentage of inhibition (shaded bar). The fractions around the major active peaks (fractions 6–25 for 9-CHO PC (B) and 31–50 for egg PCOOH (C)) were developed by TLC with chloroform/methanol/water (10/5/1) twice. Aldehyde group PC was visualized by spraying Schiff's reagent (B), and the hydroperoxide group was detected by spraying potassium iodide and starch.

Oxidized PC Products That Modify LDL
Other Methods—Protein concentration was determined by the bicinchoninic acid (BCA) method (27). The level of phosphorus from PC was determined by the method of Zhou and Arthur (28). Free amino groups were measured using fluorescamine as a probe (29).

RESULTS

Determination of Antigenic Activity of Oxidized PC Derivatives—We have reported previously (19) that the epitope of the anti-OxLDL monoclonal antibody FOH1a/DLH3 resides in oxidized products of PC and that the OxPC molecules form complexes with polypeptides including apoB. A sandwich ELISA procedure that was capable of measuring nanogram quantities of copper-induced OxLDL was used for detecting antigenic OxPC in this study. In the sandwich ELISA, OxLDL, trapped by the monoclonal antibody FOH1a/DLH3 precoated onto microtiter wells, was detected using anti-human apoB antibody (20). When the OxPC prepared from 1-palmitoyl-2-linoleoyl PC was incubated with the precoated antibody before the addition of 10 ng of protein of OxLDL, the sandwich detection of OxLDL was inhibited in a dose-dependent manner (20). The antigenic activity was estimated by the sample’s ability to compete with OxLDL.

In the initial identification of the antigenic materials, several PC analogs were tested using the competition assay (Fig. 1). The addition of OxPC extracts (1 nmol of phosphorus) to the microtiter wells precoated with FOH1a/DLH3 inhibited the detection of OxLDL by approximately 65%, while untreated PC had little effect. 9-CHO PC and egg PCOOH suppressed OxLDL binding to the monoclonal antibody. Some other analogs, lyso-PC, PAF, and 5-COOH PC also competed moderately, while PCOOH prepared from 1-palmitoyl-2-oleoyl PC was inactive. Another PC analog containing an aldehyde group at the sn-2 position, 5-CHO PC (1 nmol) also inhibited the detection of OxLDL by 90% (data not shown).

The fact that at least a part of the antigenic products can covalently modify polypeptides (see below) suggests the possibility that the apparent competition is due to inactivation of the precoated antibody by these reactive lipids. A sandwich ELISA using DLH2, another monoclonal antibody that reacts to cross-linked proteins, is capable of detection of MDA-treated LDL in a similar procedure. The addition of 9-CHO PC to the wells precoated with DLH2, however, did not show any effect on the binding of DLH2 with MDA-treated LDL (data not shown), suggesting that the decrease in OxLDL detection reflects the recognition of OxPC products by FOH1a/DLH3.

The recognition of 9-CHO PC and egg PCOOH by the monoclonal antibody was confirmed by comparing the retention times of these analogs and reactivities in the sandwich ELISA assay. When the 9-CHO PC was subjected to reversed phase HPLC, the UV absorbance at 206 nm, as well as the inhibitory activity, was eluted in fractions 15 and 16 (Fig. 2A). When the fractions around this peak were developed by TLC and sprayed with Schiff’s reagent to visualize the aldehyde group, positive bands were observed in fractions 15 and 16 (Fig. 2B). The inhibitory activity of egg PCOOH was eluted in fractions 40 and 41, and hydroperoxide was detected in the same fractions by spraying with potassium iodide and starch solutions (Fig. 2, C and D). Since the egg PCOOH was prepared from egg PC by photooxidation, hydroperoxides of 1-palmitoyl-2-linoleoyl PC would be expected to be the major components, although in fact it comprises several molecular species and their isomers. These results suggest that PC derivatives containing an aldehyde and a hydroperoxy group are recognized by the monoclonal antibody and that such OxPC products may at least in part be responsible for the antigenicity of OxPC and OxLDL to the antibody.

Isolation and Structural Analysis of the Antigenic OxPC Products—1-Palmitoyl-2-linoleoyl PC was used as a starting material for the isolation of antigenic OxPC products. The reason for this is 2-fold. First, human plasma PC has a higher content of linoleic acid than arachidonic acid (32), and second, the resulting oxidation products from PC containing linoleic acid are more easily separated and analyzed than those of arachidonic acid. OxPC prepared from 1-palmitoyl-2-linoleoyl PC was fractionated on a semipreparative straight phase

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2 H. Itabe, S. Jimi, S. Kamimura, K. Suzuki, N. Uesugi, T. Imanaka, H. Shijo, and T. Takano, submitted for publication.
HPLC (Fig. 3). Under these conditions, authentic 1-palmitoyl-2-linoleoyl PC was eluted at 13 min. Several peaks of antigenic activity were eluted between 18 and 36 min, whereas 75% of the phosphorus was eluted in the peak at 13.6 min. Four active fractions (fractions I–IV) were recovered and pooled as indicated by the horizontal bars in Fig. 3.

Fractions I–IV were then further fractionated on a reverse phase HPLC (Fig. 4). The authentic 1-palmitoyl-2-linoleoyl PC and 1-oleoyl lyso-PC eluted at 54 and 11 min, respectively, under the conditions used. The antigenic activity eluted in each fraction was tested, and the major activities were found at 32 min (RP peak I), 28 and 31 min (RP peaks IIa and IIb), 14 and 15 min (RP peaks IIIa and IIIb), and 13 min (RP peak IV), respectively. The peaks IIIa and IIIb were pooled at this stage, and rechromatographed. The recoveries and specific activities of these peaks are shown in Table I. The antigenic activity is expressed in arbitrary units, which represent the activity required to induce 50% inhibition of the ELISA assay of OxLDL. The specific activities of the authentic 9-CHO PC and the egg PCOOH were approximately 25 and 8 units/nmol, respectively, so that the peaks recovered from the reverse phase HPLC were as active as these analogs. These RP peaks were purified by rechromatography two times on the same ODS column using another solvent system, in which methanol was replaced by methanol/acetonitrile (7/3) prior to mass spectrometric analysis. In the rechromatography steps, we tried to eliminate even small shoulders and tailing areas to make the samples as pure as possible.

These purified peaks were then subjected to laser desorption mass spectrometry. The soft ionization of nonvolatile materials by laser beam allows high recovery of the (M + H)$^+$ ion and is a powerful tool for the estimation of molecular weight. As shown in Fig. 5, A and B, 1-palmitoyl-2-linoleoyl PC (calculated $M_r = 758.1$) and 1-palmitoyl-2-(9-oxononanoyl) PC ($M_r = 649.9$) gave clear signals at $m/z = 758.8$ and 650.7, which correspond to the (M + H)$^+$ ion species. The signal at 781.0 appears to be the (M + Na)$^+$ ion that is 23 larger than the original molecule. It is known that the (M + Na)$^+$ is often observed by laser desorption mass spectrometry analysis, since the sodium atom could be an impurity derived from glassware. Each sample was analyzed repeatedly, and the most prominent and constant signals observed were approximately 789 (peak I), 791 (peak IIa), 789 (peak IIb), 636 (peak IIIa), and 650 (peak IIIb), respectively (Fig. 5, C–G). The molecular weights of these products were estimated to be 1 smaller than these figures. Some minor peaks in the figure (550.8, 945.0, 685.3, and 746.3) were unknown, and they were not reproducibly detected in repeated analyses. Peak IV is lost in the final steps, since no significant signal and no antigenic activity were observed.

The molecular weight as well as the retention time on the reverse phase HPLC of the peak IIIb agreed with those of the standard 9-CHO PC (see Figs. 2A, 3C, 5B, and 5F), suggesting that peak IIIb is 9-CHO PC. The molecular weight of peak IIIa was approximately 635, which is 14 smaller than 9-CHO PC, suggesting that the structure is 8-oxooctanoyl PC. The peak IIIa eluted at 14 min on HPLC, slightly earlier than the standard 9-CHO PC (see Figs. 2A and 4D). The peak at $m/z = 496.4$ corresponds to that of 1-palmitoyl lyso-PC, suggesting that peak IIIa contains a 1-palmitoyl glycerophosphocholine backbone.

The estimated molecular weight of the peak IIa, 790, was 32 larger than that of the original PC, suggesting the presence of two additional oxygen atoms. Two of the typical oxidation products, a hydroperoxy derivative and a dihydroxy derivative, both...
have similar molecular weights. Peak IIa, however, could be a PC species containing two hydroxyl groups, since the authentic egg PCOOH eluted much later than the peak IIa on the same HPLC column (see Figs. 2B and 4B). Formation of 9,14- and 8,13-dihydroxyoctadienoic acid during autoxidation of linoleic acid was reported (30). Methoxy and ketohydroxy derivatives of PC are candidates for peaks I and IIb, as judged from their molecular weight. The similarity of the retention times on the HPLC and the molecular masses suggest that these two peaks may be the same products or isomers.

**OxPC Products That Form Complexes with Proteins**—It was shown in our earlier study that the antigenic products of this antibody could form complexes with proteins. To investigate which peaks of OxPC were responsible for complex formation, antigenic products extractable from the OxPC prepared in the presence of BSA were analyzed on a straight phase HPLC. Since an analytical column was used in this experiment, the retention times of the major peaks were not exactly the same as in Fig. 3. The inhibitory activities of OxPC prepared without BSA were eluted between standard PC and lyso-PC, and this time the active fractions were marked as I, II, III, and IV, as indicated by horizontal bars in Fig. 6. When the OxPC prepared in the presence of BSA was chromatographed, the activity in the fractions III and IV was significantly decreased (Fig. 6B). It is well known that the number of lysine residues of apoB is reduced upon oxidation of LDL (17). To test the possibility that the OxPC products in fractions III and IV react with lysine residues of BSA, PC was oxidized in the presence of MDA-treated BSA whose lysine residues had already been modified by malondialdehyde. The amino group content of this MDA-treated BSA whose lysine residues had been modified by malondialdehyde was reduced by 42% of original BSA. The OxPC prepared in the presence of MDA-treated BSA contained antigenic activity in both fractions I and II and fractions III and IV, just like the OxPC prepared without BSA (Fig. 6, A and C). Reduced activity in fractions III and IV was also observed when PC was oxidized with angiotensin II or glycine (data not shown). These results suggest that some of the antigenic OxPC products form complexes with proteins, presumably through reacting with amino groups.

When 1-palmitoyl-2-14C-linoleoyl PC oxidized in the presence of BSA was subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography, a radioactive spot was observed corresponding to the position of BSA (data not shown). No radioactivity was seen in the absence of ferrous ion. This suggests that OxPC binds to BSA. To further demonstrate the complex formation of protein with OxPC, a synthetic peptide was used as a model system. A 16-mer peptide that corresponds to the C terminus of the 70-kDa peroxisomal membrane protein (PMP70-C) was purified on reverse phase HPLC. PC treated with ferrous ion and ascorbate in the presence of the purified peptide was lyophilized, and after being redissolved in a small volume of acetonitrile/water (1/1) it was directly applied to the same HPLC column. The OxPC-peptide complex was detected by a sandwich ELISA (Fig. 7). The purified peptide was eluted at 17 min, while the sandwich ELISA-positive materials were eluted at 19 and 23 min. These results strongly suggest a covalent modification of polypeptides by OxPC products.

Further studies were carried out to determine whether PC species containing aldehyde group, which are found in fraction III, are capable of forming complexes with polypeptides. The PMP70-C was incubated with either 9-CHO PC or egg PCOOH at 37°C for 3 h. As positive and negative controls, PC and the peptide were incubated in the presence and absence of ferrous ion. Incubation of the peptide simply with 9-CHO PC produced complex(es) that were detected by a sandwich ELISA using FOH1a/DLH3 and an anti-PMP70-C antiserum, although the reactivity was reduced compared with the OxPC peptide (Fig. 8). It is possible that the decrease in extracted antigenic products (fractions III and IV in Fig. 6B) might be caused by the intermediates of the radical chain reaction of PC oxidation as well as by aldehyde derivatives of PC. On the other hand egg PCOOH incubated with the peptide failed to result in complex formation.

**DISCUSSION**

In our previous study, we obtained a unique anti-OxLDL monoclonal antibody that heavily stained macrophage-derived foam cells in human atherosclerotic lesions (19). It is very important to identify which molecules the antibody reacts with, since this would be an important aid to identifying the modified structures of OxLDL and the metabolic fate of OxLDL in foam cells. In the present study, we found that several PC species were reactive to the antibody including those capable of forming complexes with polypeptides.

It is very difficult to define the exact structure of the epitope, since not only 9-CHO PC but also egg PCOOH and other several OxPC products reacted with the antibody. Sandwich ELISA detection of the 9-CHO PC-peptide complex and LDL mixed with 9-CHO PC competed with egg PCOOH (data not shown), suggesting that these molecules share the structure.

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**TABLE I**

Recovery of phosphorus and activity of the antigenic fractions separated on HPLC columns

| Purification steps and fractions | Amount of phosphorus | Dose required for 50% inhibitiona | Total activity | Specific activity | Yield % |
|----------------------------------|----------------------|----------------------------------|----------------|------------------|--------|
| OxPC extract HPLC               | 113,800              | 1.44                             | 79,028         | 0.69             | 100    |
| Straight phase HPLC Fraction I   | 5274                 | 0.90                             | 5560           | 1.11             | 7.4    |
| Fraction II                      | 2531                 | 0.25                             | 10,124         | 4.00             | 12.8   |
| Fraction III                     | 974                  | 0.040                            | 24,350         | 25.0             | 30.8   |
| Fraction IV                      | 427                  | 0.036                            | 11,861         | 27.8             | 15.0   |
| Reverse phase HPLC Peak I       | 1332                 | 0.187                            | 7123           | 5.35             | 9.0    |
| Peak IIa                         | 607                  | 0.127                            | 4780           | 7.87             | 6.0    |
| Peak IIb                         | 569                  | 0.113                            | 5035           | 8.65             | 6.4    |
| Peaks IIIa + IIb                 | 477                  | 0.046                            | 10,370         | 21.7             | 13.1   |
| Peak IV                          | 188                  | 0.057                            | 3298           | 17.5             | 4.2    |

a The antigenic activity was determined by the competition against the binding of the monoclonal antibody to standard OxLDL as described under “Experimental Procedures.”

b One unit represents the activity that inhibits the OxLDL binding to the monoclonal antibody by 50%.
Fig. 5. Laser desorption mass spectra of the purified antigenic OxPC products. Two authentic standards, 1-palmitoyl-2-linoleoyl PC (A) and 1-palmitoyl-2-(9-oxononanoyl) PC (B), and five purified antigenic products, peak I (C), peak I la (D), peak I lb (E), peak IIa (F), peak IIa (G), and peak IIIb (G) were analyzed as described under “Experimental Procedures.” Each sample was run several times, and typical spectra are shown.
required for antigen recognition and that the recognition is not likely to specific for a certain functional group at sn-2 position. The inhibition of OxLDL binding to the antibody by high doses of choline or phosphocholine suggested that the choline head group was required for antigen recognition (20). Smaller analogs with similar structures, lyso-PC, PAF, and 5-COOH PC, moderately bound to the antibody. It is likely that the antibody

Fig. 6. Some OxPC species react to BSA. 1-Palmitoyl-2-linoleoyl PC was oxidized by incubating with ferrous ion and ascorbic acid in the absence (B) or in the presence of either BSA (A) or MDA/BSA (C) at 37 °C for 3 h. These OxPC samples extracted with Bligh and Dyer partition were analyzed on an analytical silica gel HPLC column. UV detection at 206 nm was carried out (solid line). The antigenic activity in each 1-min fraction was indicated as percentage of inhibition (shaded bar). The horizontal bars roughly indicate the areas of the active fractions I and II and fractions III and IV.

Fig. 7. Elution profile of OxPC-PMP70-C peptide complexes on a reverse phase HPLC. 1-Palmitoyl-2-linoleoyl PC was oxidized by incubating with ferrous ion and ascorbic acid in the presence of a synthetic, 16-mer peptide (PMP70-C), at 37 °C for 3 h. The whole sample was dried, redissolved in 50% acetonitrile, and injected into an ODS column (TSKgel-ODS80TM). UV detection at 220 nm was carried out (solid line). The activity in each 1-min fraction detected in the sandwich ELISA using the monoclonal antibody FOH1a/DLH3 and an antisera against PMP70-C was indicated (shaded bar).

Fig. 8. Complex formation during incubation of PMP70-C with 9-CHO PC. PMP70-C was incubated with either 9-CHO PC or egg PCOOH at 37 °C for 3 h. As controls, the PMP70-C was incubated with 1-palmitoyl-2-linoleoyl PC, and ascorbic acid at 37 °C for 3 h with or without ferrous ion. The reaction mixtures (10 nmol of PC (hatched bar) or 100 nmol PC (closed bar)) were subjected to the sandwich ELISA using the monoclonal antibody FOH1a/DLH3 and an anti-PMP70-C antisera. The results are typical of four experiments.

has some selectivity to certain OxPC products, since the specific activities of the purified peaks IIIa, IIIb, and IV (approximately 20 units/nmol) were significantly higher than that of lyso-PC (approximately 0.5 unit/nmol). Taken together, recognition of OxPC products by this antibody seems to require a lyso-PC backbone, hydrophilic functions on the sn-2 acyl chain, and a short or straight sn-2-chain. The antibody recognizes OxPC-polypeptide complexes as well as the free forms of these OxPC products, probably due to the antibody’s rather broad specificity. The PCOOH from 1-palmitoyl-2-oleoyl PC was a
rather poor antigen. A large kink at the double bond in the oleate moiety of the PCOOH might cause a steric interference between the tail of the acyl chain and the antibody.

It is well known that cleavage of lipid hydroperoxides leads to aldehyde formation during lipid peroxidation. Therefore, 9-CHO PC and 5-CHO PC were expected to be formed from PC molecules containing linoleic acid and arachidonic acid, respectively. Both of these aldehyde-containing PCs were reactive to the monoclonal antibody (see Fig. 1 and “Results”). In this study we identified 9-CHO PC as one of the antigenic determinants of this antibody. In addition, we identified the formation of another aldehyde derivative, 8-CHO-PC, which is one carbon shorter than 9-CHO PC, from palmitoyl-linoleoyl PC as an antigenic product. During extensive oxidation of methyl linoleate, 8-carbon aldehyde was produced (32). Recently Kamido et al. reported the formation of 8-CHO PC and 8-CHO cholesterylester as well as 9-CHO counterparts (33). The carbon atom at position 8 could be reactive after the carbon 13 is substituted with hydroxy or hydroperoxy group. The reaction mechanism involved in producing an 8-carbon chain derivative, however, is not fully understood.

The observed molecular weights of the antigenic products suggest several possible structures. The molecular weight of peak IIa was 790, which is 32 higher than that of the original PC molecule, suggesting that this may be PCOOH or a dihydroxy-PC derivative. The possibility that it is PCOOH is unlikely, since the retention time of peak IIa (30 min) on the reversed phase HPLC was much shorter than that of PCOOH. The PCOOH standards prepared from egg PC and 1-palmitoyl-2-oleoyl PC were 41 and 42 min, respectively. A previous study in which the oxidation products of fatty acids were analyzed extensively reported the production of 9,14-dihydroxydienoyl and 8,13-dihydroxydienoyl derivatives during autoxidation of linoleic acid (34). Peaks I and IIb, with a molecular weight of 788, 30 higher than the original PC, may be ketohydroxydienoic PC or a methoxy derivative of PC. However, the methoxy-PC is likely to elute even later than PCOOH on the reverse phase HPLC. Further study is needed to confirm the structures by identifying the functional groups.

During oxidative modification of LDL, a variety of modifications are thought to take place such as a decrease in PC content and an increase in lyso-PC content, production of MDA and subsequent modification of the lysine residues of apoB, an increase in negative charge, aggregation and fragmentation of apoB, etc. (16, 17). In our present study, we found that radioactive BSA formed on SDS-polyacrylamide gel electrophoresis after oxidative treatment of [14C]PC in the presence of BSA and that antigenic OxPC-peptide complexes were separated on an HPLC column. These data confirmed that the modification of proteins with OxPC takes place. It is possible that proteins modified by OxPC species such as 5, 8, or 9-CHO PC might be formed and accumulate in the lesions. It is reported that Ox-LDL was partially resistant to lysosomal hydrolysis, while acetylated LDL was effectively hydrolyzed (35, 36). Modification of apoB by OxPC may contribute to such changes in Ox-LDL metabolism. The foam cells in human atherosclerotic lesions were stained immunohistochemically by this monoclonal antibody (18), although the distribution of apoB protein in atherosclerotic lesions is diffuse and not concentrated in foam cells (37). Macrophage-derived foamy shaped cells in human gastric lipid islands were also positive in immunohistochemical study (38). Metabolic fates of OxPC and OxPC-protein complexes in atherosclerotic lesions are to be the subject of future investigation.

Some OxPC products are biologically active; for example 5-CHO PC was isolated from oxidized PC with platelet-activating activity (39). PCs containing short chain dicarboxylic acids were isolated as PAF-like materials in bovine brain (40) and as cytotoxic oxidation products (41). OxLDL and minimally modified LDL showed a variety of biological activities on macrophages, smooth muscle cells, and endothelial cells (4–13). Murugesan et al. (7) reported that OxLDL inhibited endothelial cell migration and this activity was recovered in the lipid extract of OxLDL. The activity to enhance monocyte binding to endothelial cells of minimally modified LDL was also recovered in a phospholipid fraction (11, 12). Recently such activity of minimally modified LDL was shown to be diminished by treatment with PAF acetylhydrolase (42), which hydrolyzes not only PAF but also oxidized products of PC with short chains at sn-2-position (43, 44). The active molecules in minimally modified LDL that had been inactivated by paraoxonase, an HDL-associated esterase, appeared to be oxidized products of PC containing multiple oxygen atoms (44). Therefore, there is a possibility that the antigenic OxPC products, including aldehyde-containing ones, possess some biological functions concerned with pathological aspects of atherosclerosis. With a sensitive method for OxLDL measurement using this monoclonal antibody (20), we have observed increased oxidation levels of plasma LDL in patients of cardiovascular diseases (3).

It is clear that more work is necessary to clarify the exact structure of the antigenic OxPC products and define the epitope of the monoclonal antibody. This antibody that can detect modification of proteins with phosphatidylethanolamine derivatives, however, would be a useful tool for the further investigation of the involvement of oxidized lipids in the pathophysiology of atherosclerosis.

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