Characterization of a specific polyclonal antibody against 13-hydroperoxyoctadecadienoic acid-modified protein: formation of lipid hydroperoxide-modified apoB-100 in oxidized LDL

Yoji Kato, Yuko Makino, and Toshihiko Osawa[1]

Laboratory of Food and Biodynamics, Department of Applied Biological Sciences, Nagoya University, Nagoya, 464-01 Japan

Abstract Lipid hydroperoxide may react with protein or amino phospholipid without secondary decomposition. We prepared a polyclonal antibody to lipid hydroperoxide-modified proteins using 13S-hydroperoxy-9Z, 11E-octadecadienoic acid-modified keyhole limpet hemocyanin (13-HPODE-KLH) as immunogen. The antibody recognized 13-HPODE-modified bovine serum albumin (BSA), but not aldehyde-modified proteins, such as malondialdehyde-modified BSA. The antibody also recognized adducts derived from 13-HPODE and 13S- hydroperoxy-9Z, 11E, 15Z-octadecatrienoic acid (13-HPOTRE(α)). The oxidized α-linolenic acid- and linoleate-protein adducts were recognized by the antibody. Oxidized phospholipid-protein adducts were scarcely recognized by the antibody. However, when ester bonds of phospholipids containing linoleic acid were hydrolyzed by alkaline treatment, the cross-reactivities appeared. The result suggests that a phospholipid hydroperoxide can react with a protein directly or indirectly, and a carboxyl terminal (COOH) of the lipid in an adduct was needed as an epitope. Oxidized LDL (ox-LDL) was prepared by the incubation of LDL with copper ion or 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), and the formation of lipid hydroperoxide-modified apolipoprotein was confirmed using the antibody. A slight immunoreactivity was observed in ox-LDL without alkaline treatment. When the ox-LDL was treated with alkali to hydrolyze the ester bonds of the lipid, enhanced antigenicity appeared with time-dependency. The results suggest that lipid hydroperoxide-modified apolipoprotein was formed during the oxidation of LDL.—**Kato, Y., Y. Makino, and T. Osawa.** Characterization of a specific polyclonal antibody against 13-hydroperoxyoctadecadienoic acid-modified protein: formation of lipid hydroperoxide-modified apoB-100 in oxidized LDL. *J. Lipid Res.* 1997. **38:** 1334–1346.

Supplementary key words antibody • oxidized LDL • lipid hydroperoxide • protein modification

Lipid peroxidation probably contributes to the initiation, promotion, and/or progression of some diseases, such as atherosclerosis. The oxidation of the lipid causes the formation of secondary decomposition products including malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE). These aldehydes covalently react with protein or nucleic acid (1). The antibodies to MDA- or HNE- modified proteins have already been prepared by some workers and used for an evaluation of the oxidative modification of proteins in vivo and in vitro (2–8).

The mechanism of the reaction between lipid hydroperoxide and amine is unknown compared to that of the aldehyde-derived modification. Shimasaki, Ueta, and Privett (9) examined the modification of protein by linoleic acid hydroperoxide. The formation of lipofuscin-like fluorescent adducts has been shown as cross-linked polymers. Fruebis, Parthasarathy, and Steinberg (10) suggest that the linoleic acid hydroperoxide reacts with the polypeptide or phosphatidylethanolamine, followed by the formation of a fluorescent compound without the formation of low molecular weight aldehydes, such as HNE or MDA. Hidalgo and Zamora

Abbreviations: MDA, malondialdehyde; HNE, 4-hydroxy-2-nonenal; LDL, low density lipoprotein; ox-LDL, oxidized LDL; KLH, keyhole limpet hemocyanin; 9-HPODE, 9S-hydroperoxy-10E, 12Z-octadecadienoic acid; 13-HPOTRE (α), 13S-hydroperoxy-9Z, 11E, 15Z-octadecatrienoic acid; 13-HPOTRE (γ), 13S-hydroperoxy-6Z, 9Z, 11E-octadecatrienoic acid; 13-HPODE, 13S-hydroperoxy-9Z, 11E-octadecadienoic acid; AAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; SLO, soybean lipoxygenase; TLC, thin-layer chromatography; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid-reactive substance; TBA, thiobarbituric acid; TCA, trichloroacetic acid; BSA, bovine serum albumin; 15-HPETE, 15S-hydroperoxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic acid; ox-BSA, oxidized BSA; ELISA, enzyme-linked immunosorbent assay; TPBS, PBS containing 0.25% Tween 20; SDS-PAGE, sodium dodecyl sulfate- polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TTBS, Tris-buffered saline containing 0.25% Tween- 20; AGE, advanced glycation end products; PC, phosphatidylcholine; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol.

[1]To whom correspondence should be addressed.

(11) reported the production of long-chain pyrrole fatty acid esters form carbonyl-amine reactions in methyl 9,10(Z)-epoxy-13-oxo-11(E)-octadecenoate or 12,13(Z)-epoxy-9-oxo(E)-octadecenoate/butylamine systems.

The preparation of the antibody to oxidized low density lipoprotein (ox-LDL) has also been done. Using the antibodies including anti-aldehyde-modified protein antibodies, the presence of oxidatively modified LDL in atherosclerotic lesions has been reported (2–5). Magil et al. (12) prepared monoclonal antibodies that recognize the fatty acid product-modified proteins. They prepared immunogens by the reaction of oxidized linoleate or arachidonate with LDL. However, the precise epitopes are not clear. Itabe et al. (13) established a monoclonal antibody that recognizes ox-LDL, using the homogenates of the human atherosclerotic plaques of the aortae as the immunogens. The epitope of the antibody is thought to be oxidized phosphatidylcholine and its complex with peptides. We assume that a lipid hydroperoxide reacts with a protein without a serious fragmentation of the fatty acid chain. A novel polyclonal antibody to lipid hydroperoxide-modified protein was prepared using 13S-hydroperoxy-9Z, 11E-octadecadienoic acid (13-HPODE)-modified keyhole limpet hemocyanin (KLH) as the immunogen. The epitopes of this antibody were characterized in detail.

EXPERIMENTAL PROCEDURES

Materials

The chemicals used were from the following sources: soybean lipoxygenase, 1-palmitoyl-2-linoleoyl-phosphatidylcholine, 1-stearoyl-2-arachidonoyl-phosphatidylcholine, 1,2-dilinoleoyl-phosphatidylcholine, α-linolenic acid, γ-linolenic acid, arachidonic acid, and lipid-free BSA were obtained from Sigma Keyhole limpet hemocyanin (KLH) was supplied by Pierce. Linoleic acid and 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) was purchased from Wako. Methyl linoleate was obtained from Nacarai. 9S-hydroperoxy-10E, 12Z-octadecadienoic acid (9-HPODE) was purchased from Cascade Biochem, Ltd. 13S-hydroperoxy-9Z, 11E, 15Z-octadecatrienoic acid (13-HPOTRE(α)) and 13S-hydroperoxy- 6Z, 9Z, 11E-octadecatrienoic acid (13-HPOTRE(γ)) were obtained from Cayman Chemical Company.

Preparation of immunogen and anti-13-HPODE-KLH antibody

13S-hydroperoxy-9Z, 11E-octadecadienoic acid (13-HPODE) was made from linoleic acid by soybean lipoxygenase (SLO). The linoleic acid (0.7 mg/ml) was dissolved in 0.1 M borate buffer (pH 9.0). SLO (7600 unit/ml) was added to the solution and reacted under O_2 with stirring at room temperature. After 90 min, the pH was adjusted to 4.0 with 0.1 M HCl for the termination of the oxidation. The peroxide formed was extracted with a 2-fold volume of chloroform-methanol 1:1. The extract was evaporated, dissolved in chloroform, and then applied to a thin-layer chromatography (TLC) plate. The chromatography was performed using a development with *n*-hexane–diethyl ether 4:6. The band of 13-HPODE was visualized by a UV lamp and scraped off. The 13-HPODE was extracted with chloroform and evaporated. The purified 13-HPODE was dissolved in ethanol and stored under argon at −70°C or −20°C before use. The identification was performed on the basis of ^{1}H-nuclear magnetic resonance spectrometry and coelution of HPLC with authentic 13-HPODE commercially obtained. The concentration of 13-HPODE was determined by absorbance at 234 nm using $\epsilon = 25000$ M^{-1} cm^{-1} (14).

The antiserum to 13-HPODE-modified protein was prepared as follows. 13-HPODE (5 mM) was incubated with 5 mg/ml keyhole limpet hemocyanin (KLH) at 37°C for 3 days in 0.1 M phosphate buffer (pH 7.4). After dialysis against phosphate-buffered saline (PBS) for 2 days at 4°C, 500 μl of the protein (in PBS) was emulsified with an equal volume of complete Freund's adjuvant to a final concentration of 0.5 mg/ml and was intramuscularly injected into a New Zealand White rabbit. After 4 weeks, 0.5 mg of 13- HPODE-modified KLH emulsified with an equal volume of incomplete adjuvant was injected as a booster.

Preparation of oxidized lipids and their characterization

Oxidized lipids were generated by thermal oxidation (12). Lipids were transferred to a glass dish open to the air and heated to 50°C for 4 days in the dark. Methanol was added to the vessel, and the oxidized lipids were transferred to vials. Aliquots of the methanol solution were used for an estimation of 2-thiobarbituric acid-reactive substances (TBARS) and lipid peroxide. The methanol was evaporated, and the lipids were dissolved in phosphate buffer (pH 7.4). The oxidized lipid-protein adduct was prepared as follows. Oxidized lipid (0.4 mM) in phosphate buffer was incubated with 200 μg/ml of lipid-free BSA for 3 days at 37°C. Aliquots of the reaction mixture were used for fluorescence measurement. The isolation of oxidized lipid-modified BSA was performed by ethanol precipitation. The oxidized lipid-modified BSA (1 ml) was precipitated by the addition of 9 ml ice- cold ethanol and kept at 4°C for 1 hr. The solution was centrifuged for 10 min, and the supernatant was discarded. Ice- cool ethanol (9 ml) was added

to the precipitate and then again centrifuged. The precipitate was dissolved in PBS and sonicated. Undissolved materials were removed by centrifugation.

Measurements of lipid peroxidation

TBARS was measured as follows. For the estimation of the thermal oxidation of the lipid, sample (2 mM lipid, 48 μl) was mixed with 30 mM BHT (2 μl), 1% TBA (50 μl), and 2.8% TCA (50 μl) in an Eppendorf tube. For measurement of the oxidation of LDL, 50 μl of the reaction mixture was mixed with 1% TBA (50 μl) and 2.8% TCA (50 μl) in an Eppendorf tube. The sample was heated at 100°C for 15 min. The tube was cooled and then centrifuged for 5 min at 4°C to remove undissolved materials. The supernatant (100 μl) was transferred to a 96-well microtiter plate and measured at 532 nm by a microplate reader (SPECTRA MAX 250, microplate spectrophotometer, Molecular Devices Corporation). The amount of TBARS was calculated from comparison with authentic malondialdehyde which was prepared by the hydrolysis of malonaldehyde bis(dimethylacetal). The lipid peroxide was measured by a determiner LPO kit (Kyowa medix), a colorimetric method based on the reaction of lipid peroxides with a methylene blue derivative in the presence of hemoglobin (15). The lipids (2 mM) were used for the determination.

Amino acid analysis

Native and 13-HPODE-modified proteins were hydrolyzed with 6 N HCl at 105°C for 24 h in vacuo. The hydrolysates were concentrated, dissolved in citrate buffer (pH 2.2), and then submitted for amino acid analysis (JLC-500/V, JEOL).

Preparation of chemically modified BSA

Malondialdehyde and 2,4-decadienal were purchased from Aldrich. Glyoxal, 1-nonanal, and 2-nonenal were obtained from Wako. Methylglyoxal and 2-hexenal were obtained from Sigma. 1-Hexanal was purchased from Nacarai. 4-Hydroxy-2-nonenal was synthesized and provided by Dr. Koji Uchida (Nagoya University). Aldehyde-modified BSAs were prepared as described (16) with some modifications as follows. Aldehyde (5 mM) and BSA (5 mg/ml) in 0.2 M phosphate buffer (pH 7.4) were mixed, and the reaction mixture was incubated for 24 h at 37°C. The reaction mixture was dialyzed against PBS at 4°C for 2 days to remove unreacted aldehyde and then used for enzyme-linked immunosorbent assay (ELISA). The degree of modification was evaluated from the loss of the amino groups determined by trinitrobenzenesulfonic acid (17). The losses of Lys residues were as follows: MDA, 53%; glyoxal, 17%; methyl glyoxal, 22%; 1-hexanal, 11%; 2-hexenal, 11%; 1-nonenal, 11%; 2-nonenal, 24%; 4-hydroxy-2-nonenal, 8.5%; 2,4-decadienal, 7.4%. Glycated BSA was prepared by the incubation of BSA (10 mg/ml) with glucose (1 M) for 225 days at 37°C in phosphate buffer, pH 7.4 (18, 19). The oxidized BSA (ox-BSA) was prepared as follows. BSA was dissolved in phosphate buffer (pH 7.4) and incubated with H_2O_2 (5 mM) in the presence of Fe(III)/EDTA (2 mM) at room temperature for 1 h. The ox-BSA was isolated by the dialysis of the reaction mixture for 3 days at 4°C. The 13-HPODE-modified BSA was prepared by the incubation of lipid-free BSA (5 mg/ml) with 13-HPODE (5 mM) in 0.1 M phosphate buffer (pH 7.4) at 37°C for 3 days, followed by dialysis against PBS. 15S-hydroperoxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic acid (15-HPETE)-modified BSA was prepared according to the method of 13-HPODE-modified protein with some modification. Briefly, arachidonic acid (0.76 mg/ml) in 0.1 M borate buffer (pH 9.0) was oxidized by soybean lipoxygenase (6500 unit/ml) for 5 min under O_2. The 15-HPETE formed was extracted and then purified by TLC. 15-HPETE-modified BSA was prepared by the incubation of BSA (5 mg/ml) with 15-HPETE (5 mM) in phosphate buffer (pH 7.4) for 3 days at 37°C. After the incubation, the reaction mixture was dialyzed against PBS for 2 days at 4°C.

Enzyme-linked immunosorbent assay (ELISA)

Cross-reactivity testing with various compounds (MDA-BSA, HNE-BSA, glycated-BSA, ox-BSA, 13-HPODE-BSA and 15-HPETE-BSA) was performed by a competitive indirect ELISA using 13-HPODE-modified BSA as a coating agent. Fifty μl of 13-HPODE-modified BSA solution (0.05 mg/ml in PBS) was dispensed into each well of the microtiter plate and kept overnight at 4°C. At the same time, competitors (50 μl) in PBS were mixed with 50 μl of antiserum (1/500) in PBS containing 1% BSA and kept overnight at 4°C. The coating solution was discarded, and the wells were then washed three times with 200 μl of PBS containing 0.05% Tween 20 (TPBS) and water. The wells were blocked with 200 μl of 4% skim milk for 1 h at 37°C with shaking. After washing, the sample (90 μl) was applied to the well and incubated with gentle shaking at 37°C for 2 h. After washing, the well was incubated with 100 μl of anti-rabbit IgG goat antibody peroxidase labeled (1/5000) in TPBS for 1 h at 37°C with shaking. After washing, 100 μl of *o*-phenylenediamine solution (*o*-phenylenediamine 5 mg/30% H_2O_2 10 μl/0.1 M citrate-phosphate buffer (pH 5.5) 10 ml) was added to each well. The plate was sometimes gently shaken at room temperature until an adequate color developed. The color development was stopped by the addition of 50 μl of 2 N H_2SO_4 to each well. The optical density at 492 nm was measured by a microplate reader. The results are expressed

as B/B_0, where B is the amount of antibody bound in the presence and B_0 in the absence of the competitor. Each point represents the mean of duplicate determinations.

For a noncompetitive indirect ELISA, a sample solution (50 μl) was added to a well and kept overnight at 4°C. After washing, the uncovered well was blocked by 4% skim milk for 1 h at 37°C and then washed. For ester bond hydrolysis, prior to blocking, 100 μl of 0.25 M NaOH was added to the well and soaked for 1 h at 37°C. An antiserum (100 μl) in PBS containing 0.5% BSA (final concentration; 1/1000) was added to the well and soaked for 2 h at 37°C. After washing, 100 μl of anti-rabbit IgG goat antibody peroxidase labeled (1/5000) was added to the well and soaked for 1 h at 37°C. After washing, a color development was performed as previously described.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) was prepared and run according to the method of Laemmli (20) using a stacking gel of 4.5%. Ten micrograms of protein was applied to each lane of the gel. The gels were stained with Coomassie Brilliant Blue. For an immunoblot analysis, the migrated protein in the gel was electroblotted to a polyvinylidene difluoride (PVDF) membrane, incubated with 4% skim milk for blocking, washed three times with Tris-buffered saline containing 0.25% Tween 20 (TTBS) for 10 min, and treated overnight with an antiserum (1/1000) in TTBS at 4°C. For hydrolysis of the ester bond, prior to blocking, the blotted membrane was immersed in 0.25 M NaOH at 37°C for 1 h with gentle shaking. This procedure was followed by an addition of horseradish peroxidase conjugated to goat anti-rabbit IgG (1/2000) and soaked for 1 h at room temperature. The binding of the second antibody to anti-13-HPODE-KLH antibody was visualized by an ECL reagent (Amersham).

Low density lipoprotein (LDL) preparation

Low density lipoprotein (LDL) was separated from human plasma by sodium bromide stepwise density gradient centrifugation. After centrifugation, the fractions with a density of 1.019–1.063 g/ml were pooled as LDL. Prior to oxidation, LDL was dialyzed against PBS at 4°C for 2 days.

Oxidation of low density lipoprotein

Copper-oxidized LDL was prepared by the incubation of LDL (0.2 mg/ml) with 50 μM copper ion in phosphate-buffered saline (PBS). The reaction was stopped by an addition of 1 mM EDTA and 10 μM butylated hydroxytoluene. Oxidation of LDL was also performed by the addition of 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) at a concentration of 0–5 mM. The reaction was carried out at 37°C for 24 h in PBS and terminated by the addition of EDTA/BHT solution as previously described. Fifty μl of ox-LDL in PBS was coated to a microtiter plate, and the formation of antigenic materials was determined by ELISA as previously described.

Reaction of lipid hydroperoxide with BSA

To investigate the effects of metal ion on the epitope formation, 13-HPODE (5 mM) was incubated with lipid-free BSA (5 mg/ml) in the absence or presence of 0.1 mM additives (Fe(II)/EDTA or DTPA). To improve the recovery of the modified protein, the lipid was extracted from the reaction mixture with chloroform–methanol 1:1. An equal amount of chloroform–methanol was added to a reaction mixture and vigorously mixed. After centrifugation, the chloroform layer was discarded and this procedure was repeated three times. Residual organic solvents were evaporated by blowing Ar gas. Protein contents were adjusted by BCA assay (Pierce) and used for a noncompetitive indirect ELISA as already described. In addition, to investigate the effects of the SH residue(s) in BSA on the formation of antigen, instead of commercially obtained lipid-free BSA, we also used partially reduced lipid-free BSA, which was prepared by the treatment of dithiothreitol (DTT) as follows. Lipid-free BSA (10 mg/ml) in water was reduced by the addition of DTT (2 mM) at room temperature for 2 h. The reaction mixture was applied to a desalting column (PD-10, Pharmacia Biotech.). The eluate between 3–6 ml was collected and dialyzed against a phosphate buffer (pH 7.4) at 4°C for 24 h. An aliquot of dialyzed sample was used for free SH residue(s) determination by Ellman's assay (21). Briefly, 0.3 ml of protein solution was added to 1.6 ml of 0.125 M phosphate buffer, pH 8.0. One hundred μl of Ellman's reagent (5,5′-dithiobis- (2-nitrobenzoic) 39.6 mg/10 ml of 50 mM phosphate buffer, pH 7.0) was added and kept for 5 min at room temperature. The amount of SH residues was calculated from a maximum extinction at 412 nm (e_{412} = 13600 M^{-1} cm^{-1}). The amount of SH residues in albumins was as follows: non-lipid BSA, 0.4 mol/mol BSA; partially reduced non-lipid BSA, 6.4 mol/mol BSA. For investigation of the effects of various lipid hydroperoxide on epitope formation, 13-HPODE, 9-HPODE, 13-HPOTRE (α), 13-HPOTRE (γ), and 15-HPETE (1 mM) were incubated with lipid-free BSA (5 mg/ml) at 37°C in a phosphate buffer. After 3 days of incubation, lipid hydroperoxide-modified BSAs were collected by ethanol precipitation as previously described.

RESULTS

Characterization of 13-HPODE-modified protein

13-HPODE (13S-hydroperoxy-9Z, 11E-octadecadienoic acid) was prepared by soybean lipoxygenase. The purified 13-HPODE was incubated at 37°C for 3 days with keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) in phosphate buffer (pH 7.4). The character of the lipid hydroperoxide-modified proteins was first investigated. The 13-HPODE-modified molecules have strong lipofuscin-like fluorescence intensity (ex. 350 nm, em. 420 nm). This agreed with the results of Shimasaki et al. (9). The results of the formation of lipofuscin-like fluorescence also coincided with the results of Freubis et al. (10). The change in amino acid composition was investigated by amino acid analysis. Specific amino acids, such as lysine and histidine residues, in 13-HPODE-modified KLH were decreased (loss of His, 15.3%; loss of Lys, 28.9%). These results may show the formation of lipid–protein adducts between these amino acids and peroxide. Not only the lysine and histidine but also the methionine in 13-HPODE-modified BSA was decreased, compared with native BSA (loss of His, 14.1%; loss of Lys, 7.6%; loss of Met, 12.7%). We cannot detect MDA- or HNE-modified derivatives in the 13-HPODE-modified BSA using anti-MDA- or HNE-modified protein antibodies (data not shown). This suggests that 13-HPODE or a molecule with a very similar structure bound to the protein, as suggested by Freubis et al. (10).

Specificity of anti-13-HPODE-KLH polyclonal antibody

Using 13-HPODE-modified KLH as an immunogen, a polyclonal antibody was prepared as described in Experimental Procedures, and the antiserum obtained was characterized by ELISA and immunoblotting. First, the formation of the antibody to 13-HPODE-modified protein was confirmed by a binding assay (indirect ELISA). As shown in **Fig. 1,** the antibody binds to the 13-HPODE-modified BSA, but not to the native BSA.

Next, oxidized BSA (modified by the Fenton System), MDA-modified BSA, HNE-modified BSA, glycated-BSA (AGE-BSA), 13-HPODE-modified BSA, and 15-HPETE-modified BSA were used as competitors, and the cross-reactivities with the polyclonal antibody were investigated by a competitive indirect ELISA. **Figure 2** shows that the 13-HPODE-modified BSA was recognized by the antibody. 15-HPETE-modified BSA also competed to a lesser extent. The formation of carboxymethyllysine during lipid peroxidation has also been reported (22, 23), and the major antigen of AGE-BSA is thought to be carboxymethyllysine residue (24). However, AGE-BSA shows no cross-reactivity. Similarly, carboxymethylated BSA, which is prepared by carboxymethylation of Lys residues in BSA with glyoxylic acid in the presence of $NaCNBH_3$ (23), is not recognized by the antibody (data not shown). The result indicates that carboxymethyllysine is not the epitope of the anti-13-HPODE- KLH antibody. Other modified BSA including HNE- and MDA-modified BSA could not inhibit the binding between the antibody and the 13-HPODE-

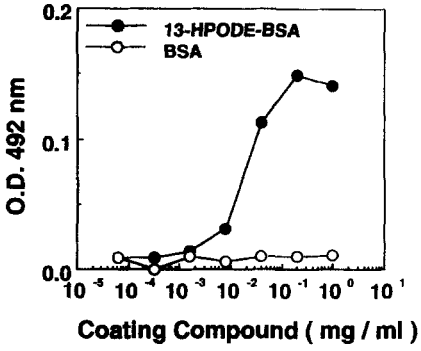

Fig. 1. Generation of antibody against 13-HPODE-modified protein. BSA (○) or 13-HPODE-BSA (●) were coated to microtiter plates. The binding activity of immune serum was investigated by indirect ELISA. The antiserum was diluted 1:1000 in PBS containing 0.5% BSA before addition to microtiter plate. The results represent the subtraction of O. D. 492 nm of preimmune serum from that of immune serum.

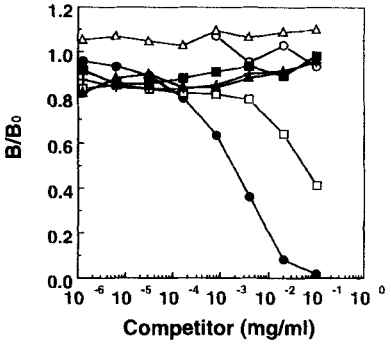

Fig. 2. Competitive indirect ELISA for chemically modified BSA. Chemically modified BSA was prepared as described in Experimental Procedures. 13-HPODE-BSA (15-HPETE-BSA) was prepared by the reaction of 13-HPODE (15-HPETE) with lipid-free BSA. MDA-BSA and HNE-BSA were made by incubation of lipid-free BSA with MDA or HNE. AGE-BSA was prepared by glycation of BSA. Ox-BSA was made by oxidation of BSA with H_2O_2-Fe/EDTA. 13-HPODE-BSA (0.05 mg/ml) was plated as antigen, and antiserum was added at a dilution of 1:1000 in the absence or presence of various concentrations of native and modified BSAs. The amount of antibody bound was detected as described, using peroxidase-labeled goat anti-rabbit IgG. The results are expressed as B/B_0, where B is the amount of antibody bound in the presence and B_0 in the absence of the competitor. Each point represents the mean of duplicate determinations. Native BSA (○); 13-HPODE-BSA (●); 15-HPETE-BSA (□); MDA-BSA (■); HNE-BSA (△); AGE-BSA (▲); Ox-BSA (+).

modified BSA coated on a solid phase. It is well known that lipid hydroperoxide decomposes and various aldehydes (such as nonenal, hexenal, or MDA) are formed. These aldehydes can react with protein. To deny the antigenicity of aldehyde-modified proteins, various aldehydes (malondialdehyde, glyoxal, methyl glyoxal, 1-hexanal, 2-hexenal, 1-nonanal, 2-nonenal, 4-hydroxy-2-nonenal, and 2,4-decadienal) were incubated with BSA, and the recognition of the aldehyde-modified BSA by the antibody was then investigated. None of aldehyde-modified BSA reacts with the antibody (data not shown).

The antigenicities of protein adducts by linoleic acid-, α-linolenic acid-, γ-linolenic acid-, and arachidonic acid-derived hydroperoxide were examined. Peroxides were incubated with BSA for 3 days at 37°C, and the formation of the antigenicity of the adducts was determined by an indirect ELISA. As shown in **Fig. 3,** 13-HPODE- and 13-HPOTRE (α)-modified BSA show immunoreactivity. The results suggest that a structure of peroxide from a terminal COOH to 13-carbon is needed for the formation of antigenicity. We tried to examine the cross-reactivity of 13-HPODE itself with the antibody. However, during competitive ELISA, 13-HPODE reacted with blocking (coating) agents from its high reactivity, and then the antigenicity of 13-HPODE could not be investigated. Next, we examined the cross-reactivity of 13-hydroxy-9Z, 11E-octadecadienoic acid (13-HODE), which is a reduced product of 13-HPODE, and linoleic acid with the antibody. 13-HODE was prepared by a reduction of 13-HPODE with $NaBH_4$ under N_2 at 4°C for 90 min. Neither 13-HODE nor linoleic acid could be recognized by the antibody (**Fig. 4**).

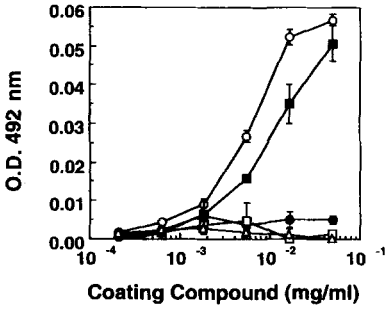

Fig. 3. Effects of peroxides on the formation of antigenicity. Peroxides (1 mM) were mixed with lipid-free BSA (5 mg/ml) and incubated for 3 days at 37°C. The peroxide-BSA adducts were isolated by ethanol precipitation and coated to microtiter plate. The antiserum was added at a dilution of 1:1000. The amount of antibody bound was detected by indirect ELISA as described, using peroxidase-labeled goat anti-rabbit IgG. Results were represented by the absorbance at 492 nm after subtraction of blank (no coating wells). The data are presented as the mean ± standard deviation of three separate determinations. 13-HPODE (○); 9-HPODE (●); 13-HPOTRE (γ) (□); 13-HPOTRE (α) (■); 15-HPETE (△).

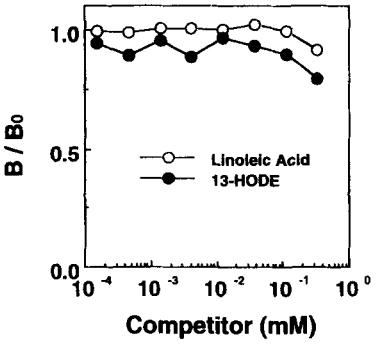

Fig. 4. Antigenicity of 13S-hydroxy-9Z, 11E-octadecadienoic acid (13-HODE). 13-HODE was prepared by reduction of 13-HPODE with $NaBH_4$. The antigenicity of 13-HODE (●) and linoleic acid (○) was estimated by competitive indirect ELISA as described in Experimental Procedures. 13-HPODE itself could not be used for ELISA because of its reactivity. The results are expressed as B/B_0, where B is the amount of antibody bound in the presence and B_0 in the absence of the competitor. Each point represents the mean of duplicate determinations.

Effect of metal ions on the formation of antigenic materials

The effects of the metal ion on the formation of antigen, i.e., 13-HPODE-BSA, were investigated. 13-HPODE was incubated with BSA in the absence or presence of a metal ion (Fe(II)/EDTA) or a metal ion chelator (DTPA). After incubation for 3 days, the adducts were obtained after lipid extraction by treatment with chloroform–methanol, and the reactivities of the adducts were examined by indirect ELISA. As shown in **Fig. 5,** the addition of Fe(II)/EDTA accelerated the formation of antigenicity. On the other hand, the metal ion chelator (DTPA) depressed the formation of antigenic materials compared to the control reaction between 13-HPODE and BSA. These results suggest that a decomposition of lipid hydroperoxide by a metal ion is required for the formation of epitopes. In addition, the effects of the SH residue(s) in BSA on the formation of antigenicity were also investigated. "Native" BSA has a single reduced cysteine (Cys 34), and the SH is considered to act as an antioxidant (25). Commercially obtained BSA is supplied in a partially oxidized form (26). The commercially obtained lipid-free BSA (0.4 mol SH/mol BSA) and the partially reduced lipid-free BSA (6.4 mol SH/mol BSA), which was prepared by the reduction of lipid- free BSA with DTT, were used for substrates and reacted with 13-HPODE. The antigenicity of the partially reduced lipid-free BSA was significantly enhanced (>2-fold compared to commercially obtained lipid-free BSA). Free SH residue(s) may become one of the good targets for 13-HPODE, and the formed adducts may become one of the epitopes of the antibody.

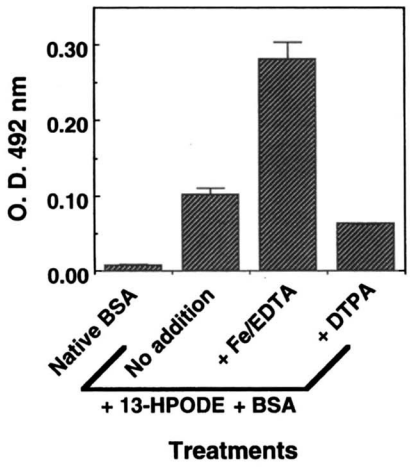

Fig. 5. Effect of metal ion on formation of 13- HPODE-BSA adducts. 13-HPODE and BSA were incubated for 3 days at 37°C in the absence or presence of 0.1 mM Fe(II)/EDTA or DTPA. The adducts were separated from lipids by chloroform–methanol and then used for indirect ELISA (0.8 μg/ml, 50 μl/well). Results were represented by the absorbance at 492 nm after subtraction of blank (no coating wells). The experiment was repeated two times separately, and similar results were obtained. The data are presented as the mean ± standard deviation of three separate determinations.

Reactivity of the antibody to oxidized fatty acid-modified BSA

Furthermore, we investigated reactivity of the antibody to oxidized lipid-modified BSA. Various lipids were oxidized at 50°C for 4 days, and the oxidized lipids were incubated with BSA at 37°C for 3 days. The progress of lipid peroxidation was evaluated by the measurement of TBARS (**Fig. 6A**) and peroxide contents (Fig. 6B). The amounts of TBARS and lipid peroxide were not so high. The low recovery of oxidation products from lipids might be due to excess oxidation of lipid. The formation of oxidized lipid–protein adducts was estimated by measurement of lipofuscin-like fluorescence (Fig. 6C). The cross- reactivities of the antibody with oxidized lipid–protein adducts were investigated by indirect ELISA (Fig. 6D). Figures 6A and 6B show that oleic acid (monounsaturated fatty acid) is less susceptible to oxidation than other polyunsaturated fatty acids. On the other hand, polyunsaturated fatty acids were oxidized and then reacted with protein molecules estimated by the increase in lipofuscin-like fluorescence. However, the extent of antigenicity was not correlated with the oxidation and increase in fluorescence. The oxidized α-linolenic acid- and linoleic acid-modified BSA show strong binding activity to the antibody (Fig. 6D, Normal). The same partial structure as that of the epitopes was probably formed in these modified BSA. The results support the cross-reactivity of 13-HPOTRE (α), which is one of the products derived from oxidized α-linolenic acid, with the antibody (see Fig. 3). Oxidized arachidonic acid- and γ-linolenic acid-modified proteins show slight cross-reactivities. Interestingly, the antibody could not react with oxidized methyl linoleate-modified protein (Fig. 6D, Normal). The results of ELISA (Fig. 6D, Normal) suggest that linoleic acid and α-linolenic acid have the ability to become the precursors of epitopes, and, therefore, the lipid hydroperoxide-modified protein has the same partial structure.

Involvement of carboxyl terminal of lipid for antigenicity

The anti-13-HPODE-KLH antibody barely recognized the oxidized methyl linoleate -, the oxidized phospholipid -, or the oxidized cholesteryl ester–protein adducts (Fig. 6D, Normal). To investigate the contribution to an immunoreactivity of terminal carbonyl of a lipid moiety in adducts, the ester bond was cleaved by treatment of oxidized lipid–protein adducts with 0.25 M NaOH. When the methyl ester bond of the methyl linoleate–protein adduct was cleaved by alkali, significant cross-reactivity was observed (Fig. 6D, +Alkali). The result suggests the need for a recognition of an adduct by the antibody and that a carboxyl terminal structure (COOH) of a lipid remained in a lipid hydroperoxide-modified protein. As shown in Fig. 6D (Normal), no reactivity of oxidized phospholipid-modified BSA, except for 1,2-dilinoleoyl-phosphatidylcholine, was observed. However, after alkaline treatment, the reactivities of phospholipids containing linoleic acid as components, such as 1,2- dilinoleoyl-phosphatidylcholine and 1-palmitoyl-2-linoleoyl- phosphatidylcholine, considerably increased (Fig. 6D, +Alkali). The results assume that phospholipid hydroperoxides were covalently attached to BSA and that a cleavage of an ester bond is required for the formation of an antigenicity of oxidized phospholipid-modified protein. The result supports no cross-reactivities of the various aldehyde-modified BSA used, including HNE- or MDA-modified BSA, which do not have a COOH structure.

Immunoblot analysis of oxidized lipid-modified BSA

The lipid–protein adducts obtained were subjected to SDS-PAGE and Western blotting. As shown in **Fig. 7A,** oxidized linoleic acid- and α-linolenic acid-modified BSA were recognized by the antibody (Fig. 7A, lanes 2 and 3). Immunoblot analysis also shows that the antigenic proteins exist in a high-molecular-weight region including the stacking gel. The methyl linoleate-BSA adduct showed its antigenicity after an alkaline treatment of the blotted membrane (Fig. 7B, lane 6). Oxidized phosphatidylcholine (containing linoleic

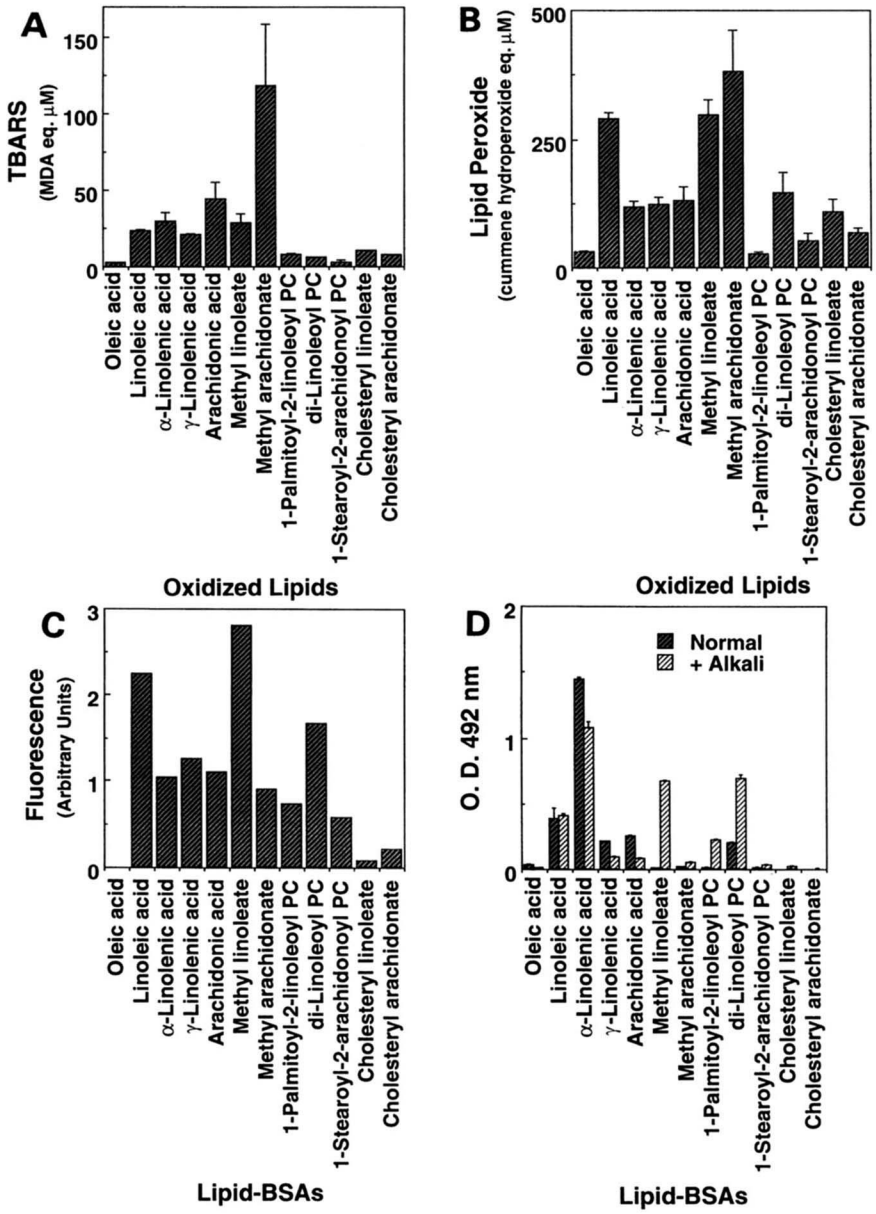

Fig. 6. Antigenicity of oxidized lipid-BSA adducts with or without alkaline treatment. Oxidized lipid was prepared by thermal oxidation. The oxidation was evaluated by formation of TBARS (A) and lipid peroxides (B) as described in Experimental Procedures. The oxidized lipid (0.4 mM) was incubated with lipid-free BSA (0.2 mg/ml) at 37°C for 3 days in phosphate buffer. The formation of lipid–protein adducts was estimated by a lipofuscin-like fluorescence (C). The lipid–protein adduct was obtained by ethanol precipitation and then used for indirect ELISA (D). The lipid–protein adducts (0.01 mg/ml, 50 μl/well) in PBS were coated to microtiter plates. Ester bond hydrolysis (+Alkali) was performed by addition of alkali (0.25 M NaOH) prior to blocking. Results were represented by the absorbance at 492 nm after subtraction of blank (no coating wells). The O. D. 492 nm of native and alkali-treated BSA is 0.069 and 0.063, respectively. The data are presented as the mean ± standard deviation of three separate determinations.

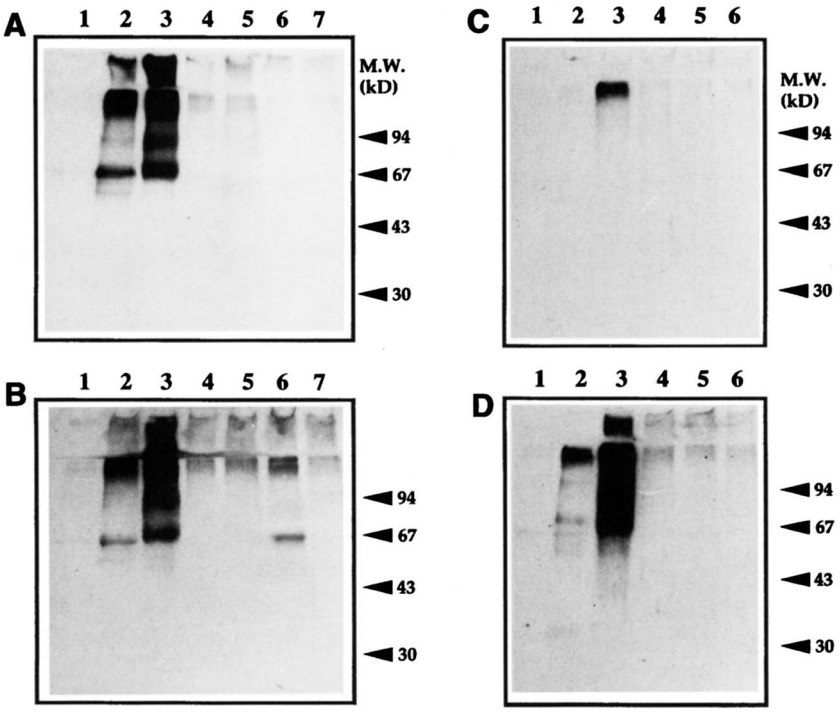

Fig. 7. Immunoblot analysis of oxidized lipid–BSA adducts. Lipid–BSA adducts were prepared as described in Fig. 6 legend. The adducts (20 μg) were subjected to SDS-PAGE and Western blotting, and the antigenic protein was detected as described in Experimental Procedures. (A, C): Normal immunoblotting. (B, D): Immunoblotting with alkaline treatment. Samples loaded onto gel were as follows: A, B: lane 1, native BSA; lane 2, oxidized linoleic acid–BSA adduct; lane 3, oxidized α-linolenic acid–BSA adduct; lane 4, oxidized γ-linolenic acid–BSA adduct; lane 5, oxidized arachidonic acid–BSA adduct; lane 6, oxidized methyl linoleate–BSA adduct; lane 7, oxidized methyl arachidonate–BSA adduct. C, D: lane 1, native BSA; lane 2, oxidized 1-palmitoyl-2-linoleoyl-PC–BSA adduct; lane 3, oxidized 1,2-dilinoleoyl-PC–BSA adduct; lane 4, oxidized 1-stearoyl-2-arachidonoyl-PC–BSA adduct; lane 5, oxidized cholesteryl linoleate–BSA adduct; lane 6, oxidized cholesteryl arachidonate–BSA adduct.

acid)-modified BSA also showed considerable increase in the visualized bands by alkaline treatment (Fig. 7D, lanes 2 and 3).

Formation of lipid hydroperoxide-modified apoprotein in oxidized LDL

It is thought that the oxidation of LDL may contribute to atherosclerosis. During the oxidation of LDL, the derivatization of amino acids in apoB-100 by lipid hydroperoxide decomposition products has been reported (17). These modifications may accelerate deposition of macrophages, and then the events can possibly lead to a conversion of macrophages to foam cells. However, the formation of lipid hydroperoxide-modified protein in ox-LDL is not known. We then examined an immunochemical detection of adducts from ox-LDL. During incubation with copper ion, lipid peroxidation proceeded as assessed by an increase in TBARS and lipid peroxide (**Figs. 8A and 8B**). Without alkaline treatment, the formation of lipid hydroperoxide-modified protein was slightly observed by ELISA (Fig. 8C, Normal). The results probably show that lipid hydroperoxides (derived free fatty acid) covalently bound to the apoprotein B-100 during lipid peroxidation in the LDL particle. As shown in Fig. 8C (+Alkali), a considerable enhancement of antigenicity was observed by alkaline treatment. A similar result of alkaline enhancement was also observed using another modality of LDL oxidation, an azo-initiator, 2,2′-azobis(2-amidinopropane)dihydrochloride (**Figs. 9A and 9B**). The results suggest that lipid hydroperoxide-modified apoproteins are formed in ox-LDL.

DISCUSSION

Several lines of evidence indicate that macrophages are the precursors of the lipid-laden foam cells that characterize early atherosclerotic lesion (27–30). The mechanism by which these cells accumulate excess lipid in the artery wall in vivo has not been determined. Macrophage may accumulate excess lipid when exposed to modified LDL, such as oxidatively modified LDL (31, 32). In vivo, the modification of LDL is probably derived from the oxidation of the lipid accompanied by derivatization of the lysine residues of apoprotein B-100 (17). However, the nature of in vivo oxidant(s) remains obscure (31). 15-Lipoxygenase, which is able to oxidize the LDL molecule in vitro (33–36), was detected in the atherosclerotic lesion of the Watanabe heritable hyperlipidemic (WHHL) rabbit or human by immunohistochemistry or in situ hybridization (37, 38).

During the oxidative degradation of polyunsaturated fatty acid, a variety of reactive aldehyde products are formed, and these are capable of covalently attaching to the protein. Malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are two examples of such products (1). The treatment of protein or amino acids with MDA results in an inactivation of the enzyme (39), cross-link-

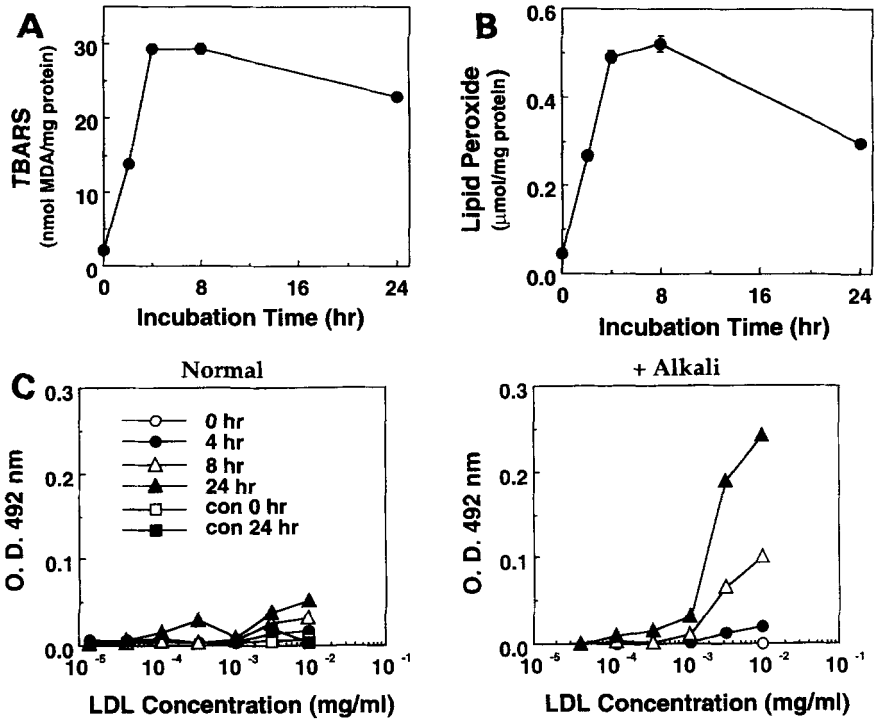

Fig. 8. Time-dependent formation of antigenic materials in copper-oxidized LDL. Oxidized LDL was prepared by incubation of LDL with copper ion. The reaction was terminated by the addition of EDTA and BHT. The oxidation of LDL was estimated by the formation of TBARS (A) and lipid peroxide (B). The amount of lipid peroxide was expressed as the equivalent of cummene hydroperoxide. The sample was coated to microtiter plate, and the formation of antigenic materials was evaluated by indirect ELISA with or without alkaline treatment (C). Results were represented by the absorbance at 492 nm after subtraction of blank (no coating wells).

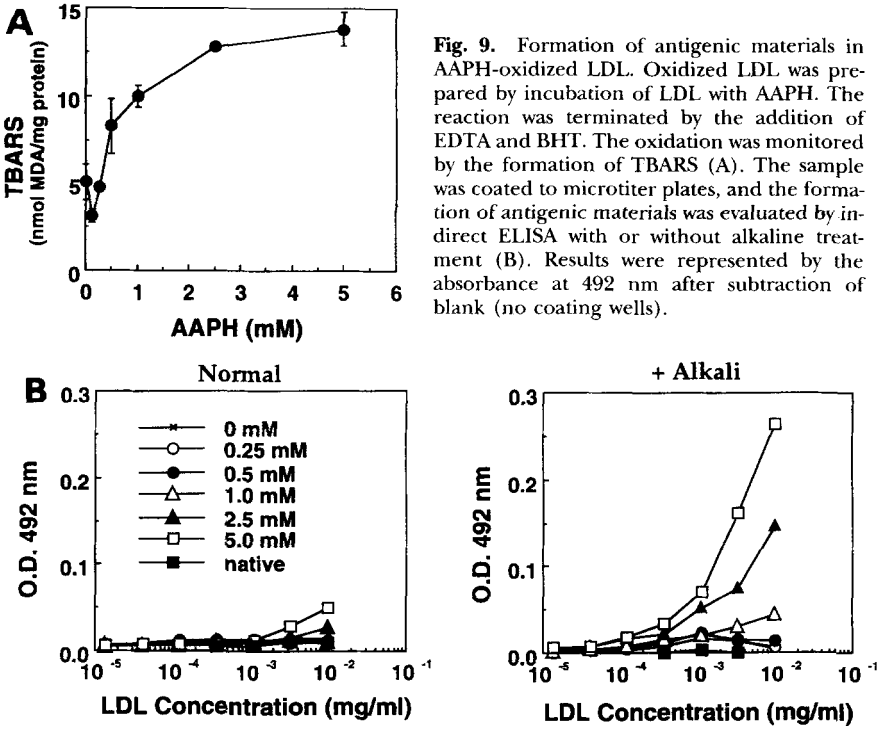

Fig. 9. Formation of antigenic materials in AAPH-oxidized LDL. Oxidized LDL was prepared by incubation of LDL with AAPH. The reaction was terminated by the addition of EDTA and BHT. The oxidation was monitored by the formation of TBARS (A). The sample was coated to microtiter plates, and the formation of antigenic materials was evaluated by indirect ELISA with or without alkaline treatment (B). Results were represented by the absorbance at 492 nm after subtraction of blank (no coating wells).

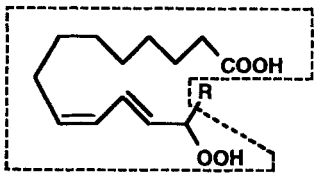

Fig. 10. Proposed essential structure of precursor for epitope formation.

ing (40), fluorescence formation (41), and amino acid modifications (40, 41). MDA reacts with ϵ-amino groups in protein and forms a 1:1 adduct (aminopropenal), 1:2 adduct (amino-imino-propen), and dihydropyridine structure. HNE treatment on LDL causes cross-linking and amino acids losses of apolipoprotein B-100 (42). HNE reacts with the ϵ-amino groups of lysine, sulfhydryl groups of cysteine, and imidazole groups of histidine through a Michael-type addition (1, 43). Anti-aldehyde-modified protein antibodies were widely used for the detection of lipid peroxidation in vitro and in vivo (2–9, 25, 39, 40, 44–46). Haberland, Fang, and Cheng (2) and Palinski et al. (4) reported evidences for the presence of aldehyde-modified LDL in the atherosclerotic lesions of rabbit and/or human. The precise structures of the epitopes for these antibodies have been gradually investigated (46). Lipid hydroperoxide can react with a protein with an intact form (10). Magil et al. (12) reported on the preparation of antibodies that recognize oxidized linoleic acid- and arachidonic acid-modified LDL, but the precise epitopes are not known. We then decided on the preparation and characterization of an antibody that recognizes lipid hydroperoxide-modified proteins.

13-HPODE-modified KLH was used for an immunogen, and an antiserum was prepared by an injection to a rabbit. The serum obtained was shown to react strongly with 13-HPODE-modified BSA, but not native BSA (Fig. 1). The antibody specifically recognizes the 13-HPODE- and 13-HPOTRE (α)-modified protein (Fig. 4). 13-HPOTRE(α) is one of the oxygenated products of α-linolenic acid. The structures of these two peroxides (13-HPODE and 13-HPOTRE (α)) contain the same structure from a terminal COOH to a C13 carbon. The result suggests that the lipid-derived part of epitopes has a terminal carbonyl. In addition, the lipid hydroperoxide-modified proteins formed do not have the structure of peroxide (–OOH) because the adducts cannot react with the lipid peroxide detection reagent (data not shown). The cross-reactivity of 13-HPODE with the antibody could not be investigated by a competitive indirect ELISA because of its high reactivity (data not shown). 13-HODE, a reduced product of 13-HPODE, is not recognized by the antibody (Fig. 4). The result suggests that the antibody probably recognizes an adduct with amine as well as the peroxidized lipid-derived structure. The specificity to 13-HPOTRE (α) is supported by the considerable cross-reactivity of oxidized α-linolenic acid–protein adducts with the antibody (Fig. 6). This antibody could not recognize various aldehyde-modified proteins. Judging from these results, the antibody has a novel and unique character.

It is well-known that lipoxygenases were inactivated by peroxides (47–49). Kishimoto et al. (50) have reported that the molecular mass difference between the native and 15-HPETE-treated 12-lipoxygenase was close to the molecular weight of 15-HPETE. Similar result was obtained by Orning et al (51). These reports support our results of the formation of lipid hydroperoxide-modified protein without a serious decomposition of the lipid moiety.

Oxidized phospholipid (containing linoleic acid)-modified BSA shows antigenicity after the ester bond cleavage by alkali. The result suggests that *1*) terminal COOH is needed for the epitopes and *2*) phospholipid hydroperoxides are also capable of adduct formation. We also detected the formation of antigenic materials in copper- and AAPH-oxidized LDL. The antigenicity was enhanced by an ester bond cleavage. The results indicate the reaction to be as follows. Oxidation of LDL causes the formation of the hydroperoxide of free fatty acid, phospholipid, cholesteryl ester, and triglyceride. These lipid hydroperoxides react with the amines, and then lipid hydroperoxide-modified proteins are formed in ox-LDL. Among the adducts formed, the antibody recognizes 13-HPODE-derived adducts but not 9-HPODE-derived adducts. The lipid hydroperoxide can also directly react with an amine moiety of the phosphatidylethanolamine (Y. Makino and T. Osawa, unpublished results). The antigenicity in ox-LDL observed using ELISA was possibly derived from 13-HPODE-modified apoprotein and also 13-HPODE-modified phosphatidylethanolamine.

In summary, we prepared a novel polyclonal antibody to the 13-HPODE-modified protein. The antibody also recognizes oxidized α- linolenic acid- and linoleic acid-modified proteins. The recognition of the oxidized phospholipid–protein adduct by the antibody requires an ester bond cleavage of the adduct. Aldehyde-modified BSA, AGE-BSA, linoleic acid, and 13-HODE failed to react to the antibody, whereas 13-HPODE- and 13-HPOTRE (α)-modified BSA show considerable antigenicity. These results suggest the existence of an essential structure of a precursor for epitope formation (**Fig. 10**). We also detected antigenic materials in copper- and AAPH-oxidized LDL. The structural estimation of the epitopes of lipid hydroperoxide-modified protein using the antibody is now in progress in our laboratory. The presence of epitopes in vivo has to be proved. This antibody may be a useful tool for an evaluation of the lipid

hydroperoxide-derived modification of biomolecules in vivo and in vitro.

We are grateful for the helpful assistance of Katsuhiko Tokunaga, Kensuke Sakai, and Khor Ooi Tiang. We thank Dr. Koji Uchida (Nagoya University) for the helpful discussion, the gift of HNE and the antibodies to HNE or MDA-modified proteins. For the LDL preparation, we also thank Dr. Hiroaki Oda (Nagoya University).

Manuscript received 16 September 1996, in revised form 28 January 1997, and in re-revised form 20 March 1997.

REFERENCES

1. Esterbauer, H., R. J. Schaur, and H. Zollner. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biol. Med.* **11:** 81–128.
2. Haberland, M. E., D. Fong, and L. Cheng. 1988. Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. *Science.* **241:** 215–218.
3. Ylä-Herttuala, S., W. Palinski, M. E. Rosenfeld, S. Parthasarathy, T. E. Carew, S. Butler, J. L. Witztum, and D. Steinberg. 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.* **84:** 1086–1095.
4. Palinski, W., M. E. Rosenfeld, S. Ylä-Herttuala, G. C. Gurtner, S. S. Socher, S. W. Butler, S. Parthasarathy, T. E. Carew, D. Steinberg, and J. L. Witztum. 1989. Low density lipoprotein undergoes oxidative modification in vivo. *Proc. Natl. Acad. Sci. USA.* **86:** 1372–1376.
5. Rosenfeld, M. E., W. Palinski, S. Ylä-Herttuala, S. Butler, and J. L. Witztum. 1990. Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. *Arteriosclerosis.* **10:** 336–349.
6. Jürgens, G., A. Ashy, and H. Esterbauer. 1990. Detection of new epitopes formed upon oxidation of low-density lipoprotein, lipoprotein [a] and very-low-density lipoprotein. Use of an antiserum against 4-hydroxynonenal-modified low-density lipoprotein. *Biochem. J.* **265:** 605–608.
7. Palinski, W., S. Ylä-Herttuala, M. E. Rosenfeld, S. W. Butler, S. A. Socher, S. Parthasarathy, L. K. Curtiss, and J. L. Witztum. 1990. Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. *Arteriosclerosis.* **10:** 325–335.
8. Hammer, A., G. Kager, G. Dohr, H. Rabl, I. Ghassempur, and G. Jürgens. 1995. Generation, characterization, and histochemical application of monoclonal antibodies selectively recognizing oxidatively modified apoB-containing serum lipoprotein. *Arterioscler. Thromb. Vasc. Biol.* **15:** 704–713.
9. Shimasaki, H., N. Ueta, and O. S. Privett. 1982. Covalent binding of peroxidized linoleic acid to protein and amino acids as models for lipofuscin formation. *Lipids.* **17:** 878–883.
10. Fruebis, J., S. Parthasarathy, and D. Steinberg. 1992. Evidence for a concerted reaction between lipid hydroperoxides and polypeptides. *Proc. Natl. Acad. Sci. USA.* **89:** 10588–10592.
11. Hidalgo, F. J., and R. Zamora. 1995. In vitro production of long chain pyrrole fatty esters from carbonyl-amine reactions. *J. Lipid Res.* **36:** 725–735.
12. Magil, A. B., J. J. Frohlich, S. M. Innis, and U. P. Steinbrecher. 1993. Oxidized low-density lipoprotein in experimental focal glomerulosclerosis. *Kidney Int.* **43:** 1243–1250.
13. Itabe, H., E. Takeshima, H. Iwasaki, J. Kimura, Y. Yoshida, T. Imanaka, and T. Takano. 1994. A monoclonal antibody against oxidized lipoprotein recognizes foam cells in atherosclerotic lesions. Complex formation of oxidized phosphatidylcholines and polypeptides. *J. Biol. Chem.* **269:** 15274–15279.
14. Verhagen, J., A. A. Bouman, J. F. C. Vliegenthart, and J. Boldingh. 1977. Conversion of 9-D- and 13-L- hydroperoxylinolenic acids by soybean lipoxygenase-1 under anaerobic conditions. *Biochim. Biophys. Acta.* **486:** 114–120.
15. Yagi, K., K. Kiuchi, Y. Saito, A. Miike, N. Kayahara, T. Tatano, and N. Ohishi. 1986. Use of a new methylene blue derivative for the determination of lipid peroxides in foods. *Biochem. Int.* **12:** 367–371.
16. Steinberg, U. P., M. Lougheed, W.-C. Kwan, and M. Dirks. 1989. Recognition of oxidized low density lipoprotein by the scavenger receptor of macrophages results from derivatization of apolipoprotein B by products of fatty acid peroxidation. *J. Biol. Chem.* **264:** 15216–15223.
17. Steinbrecher, U. P. 1987. Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. *J. Biol. Chem.* **262:** 3603–3608.
18. Nakayama, H., S. Taneda, S. Kuwajima, S. Aoki, Y. Kuroda, K. Misawa, and S. Nakagawa. 1989. Production and characterization antibodies to advanced glycation products on proteins. *Biochem. Biophys. Res. Commun.* **162:** 740–745.
19. Horiuchi, S., N. Araki, and Y. Morino. 1991. Immunochemical approach to characterize advanced glycation end products of the Maillard reaction. *J. Biol. Chem.* **266:** 7329–7332.
20. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
21. Ellman, G. L. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82:** 70–77.
22. Fu, M.-X., J. R. Requena, A. J. Jenkins, T. J. Lyons, J. W. Baynes, and S. R. Thorpe. 1996. The advanced glycation end product, N^{ϵ}-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. *J. Biol. Chem.* **271:** 9982–9986.
23. Kato, Y., K. Tokunaga, and T. Osawa. 1996. Immunochemical detection of carboxymethylated apoB-100 in copper-oxidized LDL. *Biochem. Biophys. Res. Commun.* **226:** 923–927.
24. Reddy, S., J. Bichler, K. J. Wells-Knecht, S. R. Thorpe, and J. W. Baynes. 1995. N^{ϵ}-(carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. *Biochemistry.* **34:** 10872–10878.
25. Soriani, M., D. Pietraforte, and M. Minetti. 1994. Antioxidant potential of anaerobic human plasma: role of serum albumin and thiols as scavengers of carbon radicals. *Arch. Biochem. Biophys.* **312:** 180–188.
26. Isles, T. E., and P. C. Jocelyn. 1963. The reaction of protein thiol groups with some disulphides. *Biochem. J.* **88:** 84–88.
27. Fowler, S., H. Shio, and N. J. Haley. 1979. Characterization of lipid-laden aortic cells from cholesterol-fed rabbits. IV. Investigation of macrophage-like properties of aortic cell populations. *Lab. Invest.* **41:** 372–378.

28. Schaffner, T., K. Taylor, E. J. Bartucci, K. Fischer-Dzoga, J. H. Beeson, S. Glagov, and R. W. Wissler. 1980. Arterial foam cells with distinctive immunomorphologic and histochemical features of macrophages. *Am. J. Pathol.* **100:** 57–80.
29. Gerrity, R. G. 1981. The role of the monocyte in atherogenesis. I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am. J. Pathol.* **103:** 181–190.
30. Tsukada, T., R. Ross, and A. M. Gown. 1985. Smooth muscle cell- and macrophage-specific monoclonal antibodies in the immunocytochemical analysis of human and rabbit atherosclerotic lesions. *Arteriosclerosis.* **5:** 497a–498a.
31. Berliner, J. A., and J. W. Heinecke. 1996. The role of oxidized lipoprotein in atherogenesis. *Free Radical Biol. Med.* **20:** 707–727.
32. Steinbrecher, U. P., H. Zhang, and M. Lougheed. 1990. Role of oxidatively modified LDL in atherosclerosis. *Free Radical Biol. Med.* **9:** 155–168.
33. Kühn, H., J. Belkner, H. Suzuki, and S. Yamamoto. 1994. Oxidative modification of human lipoproteins by lipoxygenases of different positional specificities. *J. Lipid Res.* **35:** 1749–1759.
34. Ezaki, M., J. L. Witztum, and D. Steinberg. 1995. Lipoperoxides in LDL incubated with fibroblasts that overexpress 15-lipoxygenase. *J. Lipid Res.* **36:** 1996–2004.
35. Benz, D. J., M. Mol, M. Ezaki, N. Mori-Ito, I. Zelán, A. Miyanohara, T. Friedmann, S. Parthasarathy, D. Steinberg, and J. L. Witztum. 1995. Enhanced levels of lipoperoxides in low density lipoprotein incubated with murine fibroblasts expressing high levels of human 15-lipoxygenase. *J. Biol. Chem.* **270:** 5191–5197.
36. Belkner, J., R. Wiesner, J. Rathman, J. Barnett, E. Sigal, and H. Kühn. 1993. Oxygenation of lipoproteins by mammalian lipoxygenases. *Eur. J. Biochem.* **213:** 251–261.
37. Ylä-Herttuala, S., M. E. Rosenfeld, S. Parthasarathy, E. Sigal, T. Särkioja, J. L. Witztum, and D. Steinberg. 1991. Gene expression in macrophage-rich human atherosclerotic lesions. 15- Lipoxygenase and acetyl low density lipoprotein receptor messenger RNA colocalize with oxidation specific lipid–protein adducts. *J. Clin. Invest.* **87:** 1146–1152.
38. Ylä-Herttuala, S., M. E. Rosenfeld, S. Parthasarathy, C. K. Glass, E. Sigal, J. L. Witztum, and D. Steinberg. 1990. Colocalization of 15-lipoxygenase mRNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA.* **87:** 6959–6963.
39. Chio, K. S., and A. L. Tappel. 1969. Inactivation of ribonuclease and other enzymes by peroxidizing lipids and by malonaldehyde. *Biochemistry.* **8:** 2827–2832.
40. Chio, K. S., and A. L. Tappel. 1969. Synthesis and characterization of the fluorescent products derived from malonaldehyde and amino acids. *Biochemistry.* **8:** 2821–2827.
41. Kikugawa, K., Y. Machida, M. Kida, and T. Kurechi. 1981. Studies on peroxidized lipids. III. Fluorescent pigments derived from the reaction of malonaldehyde and amino acids. *Chem. Pharm. Bull.* **29:** 3003–3011.
42. Jürgens, G., J. Lang, and H. Esterbauer. 1986. Modification of human low-density lipoprotein by the lipid peroxidation product 4-hydroxynonenal. *Biochim. Biophys. Acta.* **875:** 103–114.
43. Uchida, K., and E. R. Stadtman. 1992. Modification of histidine residues in proteins by reaction with 4-hydroxynonenal. *Proc. Natl. Acad. Sci. USA.* **89:** 4544–4548.
44. Holvoet, P., G. Perez, Z. Zhao, E. Brouwers, H. Bernar, and D. Collen. 1995. Malondialdehyde-modified low density lipoproteins in patients with atherosclerotic diseases. *J. Clin. Invest.* **95:** 2611–2619.
45. Montine, T. J., V. Amarnath, M. E. Martin, W. J. Strittmatter, and D. G. Graham. 1996. E-4-hydroxy-2-nonenal is cytotoxic and cross-links cytoskeletal proteins in P19 neuroglial cultures. *Am. J. Pathol.* **148:** 89–93.
46. Uchida, K., K. Itakura, S. Kawakishi, H. Hiai, S. Toyokuni, and E. R. Stadtman. 1995. Characterization of epitopes recognized by 4-hydroxy-2-nonenal specific antibodies. *Arch. Biochem. Biophys.* **324:** 241–248.
47. Gan, Q.-F., G. L. Witkop, D. L. Sloane, K. M. Straub, and E. Sigal. 1995. Identification of a specific methionine in mammalian 15-lipoxygenase which is oxygenated by the enzyme product 13- HPODE: dissociation of sulfoxide formation from self-inactivation. *Biochemistry.* **34:** 7069–7079.
48. Lepley, R. A., and F. A. Fitzpatrick. 1994. Irreversible inactivation of 5-lipoxygenase by leukotriene A4. Characterization of product inactivation with purified enzyme and intact leukocytes. *J. Biol. Chem.* **269:** 2627–2631.
49. Härtel, B., P. Ludwig, T. Schewe, and S. M. Rapoport. 1982. Self-inactivation by 13-hydroperoxylinoleic acid and lipohydroperoxidase activity of the reticulocyte lipoxygenase. *Eur. J. Biochem.* **126:** 353–357.
50. Kishimoto, K., M. Nakamura, H. Suzuki, T. Yoshimoto, S. Yamamoto, T. Takao, Y. Shimonishi, and T. Tanabe. 1996. Suicide inactivation of porcine leukocyte 12-lipoxygenase associated with its incorporation and 15-hydroperoxy-5, 8, 11, 13-eicosatetraenoic acid derivative. *Biochim. Biophys. Acta.* **1300:** 56–62.
51. Orning, L., J. Gierse, K. Duffin, G. Bild, G. Krivi, and F. A. Fitzpatrick. 1992. Mechanism-based inactivation of leukotriene A_4 hydrolase/aminopeptidase by leukotriene A_4. Mass spectrometric and kinetic characterization. *J. Biol. Chem.* **267:** 22733–22739.