Covalent Binding of Oxidized Cholesteryl Esters to Protein

IMPLICATIONS FOR OXIDATIVE MODIFICATION OF LOW DENSITY LIPOPROTEIN AND Atherosclerosis"[5]

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It has been proposed that plasma low density lipoproteins (LDL) undergo oxidative modification before they can produce foam cells in atherosclerosis. The oxidation of LDL generates a variety of reactive and aldehydic products, which covalently bind to the LDL apolipoprotein B-100 (apoB). In the present study, to investigate the mechanisms contributing to the modification of LDL, we analyzed oxidized cholesteryl esters generated during the autoxidation of LDL and characterized their covalent binding to the lysine residues of LDL apoB. In addition, we raised a monoclonal antibody specific to a lysine-bound oxidized cholesteryl ester and determined its production in human atherosclerotic lesions. The peroxidation of LDL with Cu²⁺ produced 9-oxononanoylcholesterol (9-ONC) and 5-oxovaleroylcholesterol as the major oxidized cholesteryl esters. We observed that the levels of 9-ONC and 5-oxovaleroylcholesterol peaked at 12 h and significantly decreased thereafter. The reduction of the core aldehyde levels was accompanied by (i) the formation of free 7-ketocholesterol and 7-ketocholesteryl ester core aldehydes and (ii) an increase in the amounts of apoB-bound cholesterol and 7-ketocholesteryl, suggesting that the cholesteryl ester core aldehydes were further converted to their 7-ketocholesterol- and apoB-bound derivatives. To detect the protein-bound 9-ONC, we raised the monoclonal antibody 2A81, directed against 9-ONC-modified protein, and found that it extensively recognized protein-bound cholesteryl ester core aldehydes. Agarose gel electrophoresis followed by immunoblot analysis of the oxidized LDL clearly demonstrated the formation of antigenic structures. Furthermore, immunohistochemical analysis of the atherosclerotic lesions from the human aorta showed that immunoreactive materials with mAb 2A81 were indeed present in the lesions, in which the intense immunoreactivity was mainly located in the macrophage-derived foam cells and the thickening neointima of the arterial walls. The results of this study suggest that the binding of cholesteryl ester core aldehydes to LDL might represent the process common to the oxidative modification of lipoproteins.

Atherosclerosis is a complex vascular disorder that can lead to more serious conditions such as myocardial infarction. The events in atherosclerosis are monocyte migration from the blood stream, its differentiation into macrophages in situ, the uptake of low density lipoprotein (LDL)¹ by the macrophage scavenger receptor, transformation of the lipid-laden macrophage into foam cells, smooth muscle cell proliferation and transformation into foam cells, and thus the accumulation of foam cells leading to fatty streaks and subsequent plaque formation. Various lines of evidence indicate that an important part of the pathogenesis of atherosclerosis is the oxidative modification of plasma LDL (1, 2). It has been proposed that LDL undergoes oxidative modification before it can give rise to foam cells, the key component of the progression of atherosclerosis, and that the oxidation of LDL in vivo can be reproduced by the in vitro incubation of LDL with cultured cells, such as endothelial cells, smooth muscle cells, and macrophages, or by autoxidation catalyzed by cupric ion in the absence of cells (1). During the oxidation of LDL, the LDL molecule undergoes a large number of structural changes that alter its metabolism (1). Although the detailed mechanism for the modification of LDL has not yet been established, it is generally accepted that the primary generation of lipid hydroperoxides initiates a reaction cascade leading to rapid propagation and to amplification of the number of reactive oxygen species formed; this ultimately leads to extensive fragmentation of the fatty acid chains (3) and conversion of the LDL to a more atherogenic form (4).

Lipid peroxidation leads to the formation of a broad array of different products with diverse and powerful biological activities. Among them are a variety of different aldehydes (5). The primary products of lipid peroxidation, lipid hydroperoxides (6), can undergo carbon-carbon bond cleavage via alkoxyl radicals in the presence of transition metals, giving rise to the formation of short-chain, unesterified aldehydes (6, 7) or a second class of aldehydes still esterified to the parent lipid. These esterified aldehydes are commonly termed core aldehydes (8). Although these compounds have received less attention, the formation of phospholipid and cholesteryl ester core aldehydes during the oxidation of LDL has been demonstrated (9, 10). Due to their structural similarity to the platelet-activating factor and their rather hydrophobic nature, phospholipid core aldehydes display a number of platelet-activating factor...
related biological activities. More recently, Podrez et al. (11) characterized a structurally conserved family of oxidized phosphatidylcholines that serve as novel high affinity ligands for cells stably transfected with CD36, mediating the recognition of multiple oxidized forms of LDL, and demonstrated their formation during the oxidation of LDL by multiple distinct pathways and their participation in the CD36-mediated recognition of different forms of oxidized LDL. On the other hand, with regard to oxidized cholesteryl esters, Kamido et al. (10) isolated and identified 9-oxononanoylcholesterol (9-ONC) and 5-oxovalerylcholesterol (5-OVC) as the oxidation products of cholesteryl linoleate and cholesteryl arachidonate, respectively, in oxidized LDL. The oxidation of high density lipoprotein resulted in the formation of primarily 5-OVC and 9-ONC and, although to a lesser extent, the corresponding 7-ketocholesterol derivatives (12). In line with the potential pathophysiological role of these compounds, the cholesteryl ester and 7-ketocholesterol ester core aldehydes of varying chain lengths were identified in human atheromas (13, 14). In contrast to the phospholipid core aldehydes, however, much less is known about the formation, metabolism, and biological properties of cholesteryl ester core aldehydes.

In the present study, to investigate the mechanisms contributing to the modification of LDL, we analyzed the oxidized cholesteryl esters generated during the autoxidation of LDL and characterized their covalent binding to lysine residues of LDL apoB. In addition, based on the fact that the cholesteryl ester- and 7-ketocholesterol ester core aldehydes of varying chain length were identified in human atheromas (13, 14), we raised a monoclonal antibody directed to a lysine-bound oxidized cholesteryl ester and determined its production in human atherosclerotic lesions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cholesterol, cholesteryl linolate, cholesteryl arachidonate, cholesteryl oleate, N\(^{\text{b}}\)-benzoyl-glycyl-sarcosine, and bovine serum albumin (BSA) were obtained from Sigma. Keyhole limpet hemocyanin (KLH) was obtained from Pierce. Horseradish peroxidase-linked anti-mouse IgG immunoglobulin and ECL Western blotting detection reagents were obtained from Amersham Biosciences.

**Synthesis of 9-ONC—9-ONC** was prepared by the two-step oxidation of cholesteryl oleate as previously described (10) with some modifications. Briefly, cholesteryl oleate (325 mg, 0.5 mmol in 20 ml of tetrahydrofuran) was mixed with an equal volume of 2 m M NaCNBH\(_3\) for 1 h at room temperature. The 9-ONC-\(^{-}\)/H\(_{9262}\)-benzoyl-chol-ester reaction mixtures were treated with 50 m M NaCNBH\(_3\) for 1 h at room temperature. The elution profiles were monitored by absorbance at 240 nm.

**Modification of BSA by 9-ONC**—250 \(\mu\)l of 9-ONC dissolved in methanol (pH 7.4), sonicated for 1 min, and incubated for 4 h at room temperature. After the incubations, the reaction mixtures were treated with 50 \(\mu\)l NaCNBH\(_3\) to reduce the disulfides. The reaction mixtures were analyzed by LC-MS equipped with a Jasco Platform II-MS instrument, 0.5% ammonium hydroxide was added to the mobile phases.

**Amino Acid Analysis**—An aliquot (0.1 ml) of the protein samples incubated in the absence or presence of 9-ONC was treated with 100 \(\mu\)l of 25% (w/v) trichloroacetic acid and then extensively dialyzed against a 1000 fold volume of phosphate-buffered saline. The elution profiles were monitored by absorbance at 240 nm.

**Lipid Peroxidation Assay**—The lipoperoxidation assay was performed by incubating the emulsified cholesteryl esters with Cu\(^{2+}\) diazylated against a 1000 fold volume of phosphate-buffered saline at 4°C. The autoxidation of the cho- esteryl ester and LDL was performed by measurement of 2-thiobarbituric acid-reactive substances (TBA- \(R\)S). The reaction mixture (0.1 ml) was treated with 0.5 ml of 2.8% (w/v) trichloroacetic acid and 0.5 ml of 1% (w/v) trichloroacetic acid and 0.5 ml of sodium phosphate buffer (pH 7.4) at 37°C. Informed consent was obtained under the study approved by our institutional review board. The study was performed in accordance with the Helsinki Declaration of 1975 as revised in 1983.

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buffer (pH 7.4) at 37°C for 24 h. The 9-ONC-treated KLH was further treated with NaCNBH₃ to reductively aminate the 9-ONC-borne Schiff base adducts. Female BALB/c mice were immunized three times with the 9-ONC-modified KLH. Spleen cells from the immunized mice were fused with P3/U1 murine myeloma cells and cultured in hypoxanthine/aminopterin/thymidine selection medium. The culture supernatants of the hybridomas were screened using an enzyme-linked immunosorbent assay (ELISA), employing pairs of walls of microtiter plates on which were absorbed 9-ONC-treated BSA as the antigen (0.5 μg of protein/well).

ELISA—A 100-μl aliquot of the antigen solution was added to each well of a 96-well microtiter plate and incubated for 20 h at 4°C. The antigen solution was then removed, and the plate was washed with Tris-buffered saline (TBS) containing 10% Tween 20 (TBS/Tween). Each well was incubated with 200 μl of 1% BSA in TBS/Tween for 30 min at 37°C to block the unsaturated plastic surface. The plate was then washed once with TBS/Tween. A 100-μl aliquot of the antibody serum was added to each well and incubated for 1 h at 37°C. After discarding the supernatants and washing three times with TBS/Tween, a 100 μl of a 5 × 10⁻⁵ dilution of goat anti-mouse IgM conjugated to horseradish peroxidase in TBS/Tween was added. After incubation for 1 h at 37°C, the supernatant was discarded, and the plates were washed three times with TBS/Tween. The enzyme-linked antibody bound to the well was revealed by adding antibody solution (0.1 mg/ml in 1.2% phenylenediamine in 0.1% citrate/phosphate buffer (pH 5.0) containing 0.0055% hydrogen peroxide. The reaction was terminated by the addition of 2 M sulfuric acid, and the absorbance at 492 nm was read on a micro-ELISA plate reader.

In a competitive ELISA, a competitor was added with antibody for 20 h at 4°C to yield competitor/antibody mixtures containing antibody at 0.8 μg/ml and variable concentrations of the competitor. A 100-μl aliquot of competitor/antibody mixtures was added to each well and incubated for 1 h at 37°C. After discarding the supernatants and washing three times with TBS/Tween, the second antibody was added, and the enzyme-linked antibody bound to the well was revealed as described above. Results were expressed as the ratio B/B₀, where B represents absorbance in the presence of competitor minus background absorbance (no antibody, and B₀ is absorbance in the absence of competitor). 

Agarose Gel Electrophoresis/Immunoblot Analysis—Agarose gel electrophoresis of LDL was performed with the Helena TITAN GEL High Resolution Protein System (Helena Laboratories, Saitama, Japan). The samples were run on two separate gels. One gel was used for staining with Ponceau 7B, the other was transblotted to nitrocellulose membranes, incubated with Block Ace (40 mg/ml for blocking, washed, and treated with the primary antibody (mAb 2A81). This procedure was followed by the addition of horseradish peroxidase conjugated to a goat anti-mouse IgM and ECL reagents (Amersham Biosciences). The bands were visualized by exposure of the membranes to autoradiography film.

Immunostaining—This investigation was carried out on aortic wall samples obtained at autopsy from five patients with generalized arteriosclerosis without diabetes mellitus. Each autopsy was performed at Tokyo Women’s Medical University after the patients’ family members granted informed consent according to the established guideline. The postmortem time before starting autopsy varied from 1 to 3 h. No significant difference of morphological changes in the aorta was seen among these cases with different postmortem time. Each sample was prepared for 10% formalin-fixed, paraffin-embedded materials and for frozen materials embedded in the optimum cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) at ~ 80°C. Multiple 6-μm thick sections were cut from these paraffin-embedded and frozen materials and used for histopathological and immunohistochemical examinations. Frozen-embedded sections were deparaffinized with xylene and ethanol, rehydrated in distilled water, quenched for 10 min with 3% hydrogen peroxide for inhibiting endogenous peroxidase activity, rinsed in phosphate-buffered saline, pH 7.6, and pretreated for 30 min at room temperature with 3% nonimmune animal serum, from the same species as those producing secondary antibodies, in phosphate-buffered saline. After blocking nonspecific antibody binding, biotinylated anti-BSA immunostaining for CD68, paraffin sections were treated for 30 min at 37°C with 0.1% trypsin in phosphate-buffered saline for antigen retrieval. Frozen sections were dried, postfixed or not in 10% formalin, rehydrated, processed with the same subsequent procedures as those for paraffin sections, and pretreated with the endogenous avidin/biotin blocking kit (Vector Labs, Burlingame, CA, Vector Laboratories, Burlingame, CA, Vector Laboratories, Burlingame, CA, Vector Laboratories, Burlingame, CA). For staining of frozen sections, 1 μm EDTA was added to each solvent for removal of transition metals. These prepared sections were then incubated overnight at 4°C with mAb 2A81 at a concentration of 0.5 μg/ml and the mouse monoclonal anti-CD68 IgG antibody (KP-1; Dako, Glostrup, Denmark) at a dilution of 1:500. CD68 was used as a specific marker of macrophages. Immunoreaction was visualized by the avidin-biotin-immunoperoxidase complex method using Vectorstain ABC kit (Vector). The chromagen was 3,3′-diaminobenzidine tetrahydrochloride, and the counterstain was hematoxylin. Sections from which the primary antibodies were omitted served as negative reaction controls. An immunoperoxidase test was performed on sections incubated with mAb 2A81 pretreated with 50 μg/ml 9-ONC-conjugated BSA. Immunohistochemical localization of the immunoreaction product was then visualized by light microscopy on consecutive sections with hematoxylin-eosin or immunostained for CD68.

RESULTS

Transient Accumulation of Cholesteryl Ester Core Aldehydes during Peroxidation of Cholesteryl Esters and LDL—We analyzed the aldehydic molecules generated during the peroxidation of cholesteryl linoleate and cholesteryl arachidonate. As shown in Fig. 1A, when cholesteryl linoleate was treated with 10 μM Fe²⁺ and 1 mM ascorbate in 50 mM sodium phosphate buffer (pH 7.4), the TBARS values reached a maximum at 24 h and decreased thereafter. The LC-MS analysis following the DNPH derivatization demonstrated that, in addition to the short-chain aldehydes such as acetaldehyde, hexanal, and 4-hydroxy-2-nonenal (Fig. 1B), the metal-catalyzed peroxidation of cholesteryl linoleate mainly produced 9-ONC (Fig. 1C). Upon autoxidation of cholesteryl arachidonate, 5-OVC was detected as the major product (Fig. 1D). These core aldehydes were estimated to account for a minimum of 1–2% of the consumed cholesteryl esters.

Subsequently, we characterized the formation of these core aldehydes during the peroxidation of LDL. To efficiently induce LDL peroxidation, Cu²⁺, instead of Fe²⁺, was used as the catalyst. Both Cu²⁺ and Fe²⁺ can be used to initiate LDL oxidation in vitro, since the resulting oxidized LDL exhibits similar biological activities to that oxidized in vivo. However, Cu²⁺ is more frequently used for the in vitro LDL oxidation, because Cu²⁺ alone can effectively oxidize LDL whereas a chelating agent, such as citrate, is required for the Fe²⁺-initiated LDL oxidation (16). As shown in Fig. 2A, the TBARS values reached a maximum between 12 and 24 h. In addition, the cholesteryl esters contained in the LDL were eventually consumed, and all of them disappeared within 24 h of incubation (Fig. 2B). In parallel to the extent of the LDL oxidation, there was a progressive increase in the number of cholesteryl ester core aldehydes, 9-ONC and 5-OVC (Fig. 2C). The decrease in the cholesteryl ester concentrations closely reflected the formation of these core aldehydes. It was noted that the amounts of both core aldehydes peaked (150 nmol of 9-ONC and 25 nmol of 5-OVC per mg of LDL) at 12 h and continuously decreased thereafter (Fig. 2D). This result suggests that these cholesteryl ester core aldehydes may be the intermediates that undergo further reactions during the LDL peroxidation.

Oxidative Conversion of Cholesteryl Ester Core Aldehydes to Their 7-Ketocholesterol Derivatives—It was anticipated that the decrease in the cholesteryl ester core aldehyde concentrations during LDL peroxidation might be due to their conversion to the corresponding oxidized products, such as the 7-ketocholesterol ester core aldehydes. To assess this presumption, we examined the formation of 7-ketocholesterol and its ester core aldehydes by LC-MS following DNPH derivatization. Because the peak for the DNPH derivative of 7-ketocholesterol overlapped with the peaks for the DNPH derivatives of 5-oxovaleryl-7-ketocholesterol and 9-oxononanoyl-7-ketocholesterol (Fig. 3A), we determined these products together as the total amounts of 7-ketocholesterol and its ester core aldehydes. As shown in Fig. 3B, in contrast to the formation of TBARS and the cholesteryl ester core aldehydes (Fig. 2), the yields of 7-ketocholesterol and its ester core aldehydes were relatively low.
during the early incubation period of 8 h; however, accompanied by the decrease in the 9-ONC and 5-OVC concentrations after 12 h, the formation of 7-ketocholesterol and its ester core aldehydes was dramatically enhanced. These data suggest that the consumption of the cholesteryl ester core aldehydes closely reflects, at least in part, the oxidation of the cholesterol moieties to their corresponding 7-keto derivatives.

**Covalent Binding of Cholesteryl and 7-Ketocholesterol Ester Core Aldehydes to LDL ApoB**—An alternative mechanism for the consumption of the cholesteryl ester core aldehydes may include their covalent binding to the apoB of LDL. During the oxidation of LDL, there is a progressive decrease in the amino groups. It has been generally assumed that this reflected the conjugation of short-chain aldehydes (e.g. malondialdehyde or 4-hydroxy-2-nonenal) with the lysine ε-amino groups (17). We wanted to learn to what extent, if at all, the bound cholesteryl ester core aldehydes contributed to the blocking of the lysine ε-amino groups. To characterize the putative bound cholesteryl esters, we prepared the oxidized LDL, extracted lipids, and then treated the apolipoprotein with dilute alkali. If the linkage occurs between a fragmented side-chain fatty acid aldehyde of the oxidized cholesteryl esters and a lysine residue of apoB, saponification should result in the release of cholesterol (or oxidized cholesterol). In fact, we determined the release of

**Fig. 1. Formation of cholesterol core aldehydes during peroxidation of cholesteryl esters.** The autoxidation of cholesteryl esters was performed by incubating the emulsified 20 mM cholesteryl ester liposome with $\text{Fe}^{2+}$ (10 μM) and ascorbate (1 mM) in phosphate buffer (pH 7.4). CL, cholesteryl linoleate; AsA, ascorbate. A, formation of TBARS. ○, cholesteryl linoleate alone; ●, cholesteryl linoleate plus $\text{Fe}^{2+}$/ascorbate. B, formation of short-chain aldehydes. Lower chromatogram, cholesteryl linoleate alone; upper chromatogram, cholesteryl linoleate plus $\text{Fe}^{2+}$/ascorbate. Peak 1, acetaldehyde; peak 2, 4-hydroxy-2-nonenal; peak 3, hexanal. C, LC-MS analysis of the DNPH derivative of 9-ONC in the oxidized cholesteryl linoleate. *Bottom,* HPLC profile of the reaction mixture. *Middle,* mass spectrum of the peak eluted at 26 min in the HPLC chromatogram. *Top,* selected ion current chromatograms obtained from the LC-MS analysis monitored with $m/z$ 719 for the DNPH derivative of 9-ONC. D, LC-MS analysis of the DNPH derivative of 5-OVC in the oxidized cholesteryl arachidonate. *Bottom,* mass spectrum of the peak eluted at 21 min in the HPLC chromatogram. *Middle,* HPLC profile of the reaction mixture. *Top,* selected ion current chromatograms obtained from the LC-MS analysis monitored with $m/z$ 663 for the DNPH derivative of 5-OVC.
primarily cholesterol (Fig. 4A) and 7-ketocholesterol (Fig. 4B). Furthermore, the amount of the released cholesterol increased right after the beginning of the incubation and reached a plateau at 24 h, whereas the release of 7-ketocholesterol was observed at and after 8 h of incubation (Fig. 4C). These data establish the fact that the cholesteryl ester core aldehydes could be covalently linked to apoB.

9-ONC-Lysine Schiff Base Adduct—9-ONC was presumed to be covalently bound to the lysine residues of the protein via a Schiff base linkage. Indeed, upon incubation of BSA with 9-ONC, the loss of the lysine residues was quite significant (Fig. 5A). In an attempt to detect the 9-ONC-lysine Schiff base adduct in protein by the amino acid analysis, the authentic 9-ONC-lysine adduct was prepared by incubating N°-benzoyl-glycyl-lysine with 9-ONC, reductively aminating it with NaCNBH₃, and then characterizing it by LC-MS and amino acid analyses. As shown in Fig. 5B, the LC-MS analysis of the major product eluted at 21 min showed a pseudomolecular ion peak at m/z 832.1 (M + H)⁺, corresponding to the reduced form of the 9-ONC-lysine adduct. Upon amino acid analysis of the reaction mixtures, we detected a new peak, which might have originated from the 9-ONC-lysine adduct (data not shown). However, it was found that the peak of 9-ONC-lysine completely overlapped that of histidine in the amino acid analysis.

Preparation of Monoclonal Antibody against 9-ONC-Lysine Schiff Base Adduct—As an alternative approach for the detection of the 9-ONC-lysine Schiff base adduct, we attempted to raise the monoclonal antibody specific to the 9-ONC-modified protein. Mice were immunized with the 9-ONC-modified KLH, and, during the preparation of the monoclonal antibodies, hybridomas were selected by the reactivities of the culture supernatant to the 9-ONC-modified BSA. We finally obtained one clone (2A81), which showed the most distinctive recognition of the 9-ONC-modified BSA. The isotype on the monoclonal antibody was IgM with a κ light chain. Upon incubation with BSA, 9-ONC generated immunoreactive materials with mAb 2A81 in time- and dose-dependent manners (Figs. 6, A and B). Despite an extensive screening of the hybridomas, which produce monoclonal antibodies specific to the 9-ONC-modified BSA, it is still conceivable that the antibody...
recognizes epitopes originating from other lipid peroxidation products. Hence, we examined the immunoreactivity of the antibody to the aldehyde-treated BSA by direct ELISA and found that, among the aldehydes tested, 9-ONC was the only source of immunoreactive materials generated in the protein (Fig. 6C). In addition, the monoclonal antibody was found to recognize the protein-bound 9-ONC reductively aminated with NaCNBH$_3$, indicating that the Schiff base double bond might not be essential for the antibody recognition.

Formation of Antigenic Structures in the Protein Exposed to the Iron/Ascorbate-mediated Oxidation of Cholesteryl Esters and in Oxidized LDL—To examine whether the peroxidation of cholesteryl esters could generate antigenic structures in protein, we incubated the cholesteryl esters (cholesteryl linoleate, cholesteryl arachidonate, and cholesteryl oleate) with an iron/ascorbate-mediated free radical generating system in the presence of BSA and examined their formations in the modified

![Graph A](image)

**Fig. 3.** Formation of 7-ketocholesterol and its core aldehydes during the peroxidation of LDL. The oxidation of LDL was performed by incubating 0.5 mg of LDL with CuSO$_4$ (5 μM) in 1 ml of 50 mM sodium phosphate buffer (pH 7.4) at 37 °C. A, selected ion current chromatograms obtained from LC-MS analysis monitored with m/z 579, 677, and 733 for the DNPH derivatives of 7-ketocholesterol (7KC), 5-oxovaleroyl-7-ketocholesterol, and 9-oxononanoyl-7-ketocholesterol, respectively. B, time-dependent changes in the total amount of 7-ketocholesterol and its core aldehydes.

![Graph B](image)

**Fig. 4.** Covalent binding of cholesteryl and 7-ketocholesterol ester core aldehydes to LDL apoB. LDL (0.5 mg) was incubated with 5 μM Cu$^{2+}$ in 1 ml of 50 mM sodium phosphate buffer (pH 7.4) at 37 °C. After delipidation, the apoB samples were treated with dilute alkali, and the cholesterol and 7-ketocholesterol (7KC) released from the apoB were analyzed by HPLC. A, HPLC analysis of released cholesterol. The LDL treated with Cu$^{2+}$ for 48 h was used for the analysis. oxLDL, oxidized LDL. **Top,** with alkali treatment. **Middle,** without alkali treatment. **Bottom,** cholesterol standard. B, HPLC analysis of released 7-ketocholesterol. The LDL treated with Cu$^{2+}$ for 48 h was used for the analysis. **Top,** with alkali treatment. **Middle,** without alkali treatment. **Bottom,** 7-ketocholesterol standard. C, time-dependent changes in the apoB-bound cholesterol and 7-ketocholesterol levels. ●, cholesterol; ○, 7-ketocholesterol.
proteins by ELISA. As shown in Fig. 7A, the antibody showed a significant immunoreactivity with the protein exposed not only to the oxidized cholesteryl linoleate but also to the oxidized cholesteryl arachidonate. In addition, the competitive ELISA analysis showed that the antibody binding to the 9-ONC-modified protein was not inhibited by free 9-ONC and 7-ketocholesterol but significantly inhibited by the protein- and peptide-bound 9-ONC (Fig. 7, B and C). These data suggest that the antibody extensively recognizes the protein-bound cholesteryl ester core aldehydes, including 9-ONC-lysine and 5-OVC-lysine.

To determine whether antigenic structures recognized by mAb 2A81 are formed in oxidized LDL, LDL treated with 5 mM Cu²⁺ was subjected to an agarose gel electrophoresis followed by immunoblot analysis with mAb 2A81. As shown in Fig. 7D, the native form of the LDL appeared as a single protein band that was readily visualized by Fat Red 7B staining; however, the LDL incubated with 5 μM Cu²⁺ exhibited enhanced anodic mobility compared with the native LDL, indicating an in-
The formation of lipid peroxidation products bound to proteins in vascular lesions, such as the atherosclerotic lesion, is a phenomenon common in most, if not all, types of vascular damage associated with oxidative stress. The possibility that aldehydic molecules generated from lipid peroxidation play a role in the pathogenesis of atherosclerosis is suggested by the facts that (i) the level of reactive aldehydes increases in plasma in relation to extensive aortic atherosclerosis, (ii) high concentrations of aldehydes can be generated during the oxidation of LDL phospholipids, (iii) the structural and functional changes associated with the in vitro oxidation of LDL can also be produced by the direct interaction of LDL with aldehydes, and (iv) the reaction of aldehydes with a critical number of lysine residues of the LDL apoB produces internalization by the scavenger receptor of human monocyte-macrophages and subsequent intracellular accumulation of the lipoprotein-derived cholesteryl ester. On the other hand, it has been shown that atherosclerotic lesions of varying severity from the human aorta contain material recognized by antibodies raised against protein adducts with aldehydes. Haberland et al. (18) and Paliński et al. (19) demonstrated that monoclonal antibodies directed against MDA-modified LDL bind to epitopes in atherosclerotic lesions. They proposed the hypothesis that the formation of MDA and modification of the lysine residues of LDL apoB occur in vivo as a prerequisite to the formation of arterial foam cells and contribute to the development of athero-

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**Fig. 7.** Formation of antigenic structures in the protein exposed to the iron/ascorbate-mediated oxidation of cholesteryl esters and in oxidized LDL. A, formation of antigenic structures in the protein exposed to the iron-catalyzed autoxidation of cholesteryl esters. BSA (0.5 mg/ml) was incubated with 20 mM cholesteryl ester (cholesteryl linoleate (CL), cholesteryl arachidonate (CA), or cholesteryl oleate (CO)) in the presence of Fe2+ (10 μM) and ascorbate (AsA; 1 mM) for 24 h in 50 mM sodium phosphate buffer (pH 7.4). B, competitive ELISA with free 9-ONC, 7-ketocholesterol, and protein-bound 9-ONC. The protein-bound 9-ONC was prepared by incubating BSA (0.5 mg/ml) with 9-ONC (5 mM) in 50 mM sodium phosphate buffer (pH 7.4) for 24 h at 37 °C. C, competitive ELISA with free 9-ONC and peptide-bound 9-ONC. The 9-ONC-modified peptide was prepared by incubating 5 mM N-benzyloxy-glycyl-lysine with 5 mM 9-ONC in 50 mM sodium phosphate buffer (pH 7.4) for 24 h at 37 °C. D, agarose gel electrophoresis/immunoblot analysis of oxidized LDL. The oxidation of LDL was performed by incubating 0.5 mg of LDL with CuSO4 (5 mM) in 1 ml of 50 mM sodium phosphate buffer (pH 7.4) at 37 °C. Top, agarose gel electrophoresis; bottom, agarose gel electrophoresis/immunoblot analysis with mAb 2A81.

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increased negative charge of the molecule due probably to the modification of the ε-amino group of the lysine residues (upper panel). An agarose gel electrophoresis/immunoblot analysis of the Cu2+-oxidized LDL using mAb 2A81 revealed the formation of immunoreactive materials, which were not detected in the native LDL (lower panel).

**Localization of Antigenic Structures in Human Atherosclerotic Lesions**—The recent findings that the level of 9-ONC increases in the plasma of patients with atherosclerosis (13, 14) suggested that the 9-ONC-lysine Schiff base adduct was most likely accumulated in an atherosclerotic lesion. Hence, atherosclerotic aortas were immunohistochemically examined for protein-bound cholesteryl ester core aldehydes using mAb 2A81. As shown in Fig. 8, the macrophages appearing in the atherosclerotic plaques were identified in hematoxylin-eosin-stained (A) or CD68-immunostained sections (B). The cytoplasm in most of the foamy or spindle macrophages was immunoreactive with mAb 2A81 (Fig. 8C). No immunoreaction product deposits were detectable in a section incubated with mAb 2A81 preabsorbed with the specific antigen (Fig. 8D). There was no significant difference in the localization of the antigenic structures between the paraffin-embedded and frozen sections (Supplementary Figs. 1 and 2). In addition, neither formalin postfixation nor EDTA addition had any effect on the immunohistochemical results (Supplementary Figs. 1 and 2). These data suggest that the generation of antigenic structures recognized by the antibody during the dehydration, embedding, rehydration, and staining processes is unlikely and that the observed immunoreactivities reflect the endogenous products generated in vivo. Thus, the detection of protein-bound cholesteryl ester core aldehydes in the atherosclerotic plaques supports the notion that the reaction between oxidized cholesteryl esters and primary amines might represent a process common to the LDL modification during aging and its related diseases.

**DISCUSSION**

The formation of lipid peroxidation products bound to proteins in vascular lesions, such as the atherosclerotic lesion, is a phenomenon common in most, if not all, types of vascular damage associated with oxidative stress. The possibility that aldehydic molecules generated from lipid peroxidation play a role in the pathogenesis of atherosclerosis is suggested by the facts that (i) the level of reactive aldehydes increases in plasma in relation to extensive aortic atherosclerosis, (ii) high concentrations of aldehydes can be generated during the oxidation of LDL phospholipids, (iii) the structural and functional changes associated with the in vitro oxidation of LDL can also be produced by the direct interaction of LDL with aldehydes, and (iv) the reaction of aldehydes with a critical number of lysine residues of the LDL apoB produces internalization by the scavenger receptor of human monocyte-macrophages and subsequent intracellular accumulation of the lipoprotein-derived cholesteryl ester. On the other hand, it has been shown that atherosclerotic lesions of varying severity from the human aorta contain material recognized by antibodies raised against protein adducts with aldehydes. Haberland et al. (18) and Paliński et al. (19) demonstrated that monoclonal antibodies directed against MDA-modified LDL bind to epitopes in atherosclerotic lesions. They proposed the hypothesis that the formation of MDA and modification of the lysine residues of LDL apoB occur in vivo as a prerequisite to the formation of arterial foam cells and contribute to the development of athero-

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osclerosis. The participation of reactive aldehydes in atherosclerosis is also suggested by a series of immunohistochemical analyses of atherosclerotic lesions from the human aorta using various antibodies against short-chain aldehyde-amino acid adducts, such as 4-hydroxy-2-nonenal-histidine (20), MDA-lysine (21, 22), and acrolein-lysine (23), in which the immunoreactivities are commonly associated with cells, primarily macrophages. These studies provided evidence for the presence of oxidized LDL (or at least of antigens closely related to it) in arterial lesions at significant concentrations and suggested that reactive aldehydes may play a role in the formation of arterial foam cells.

It is well established that unesterified aldehydes formed during LDL oxidation can modify lysine or histidine residues of apolipoproteins by Schiff base or Michael adduct formation during LDL oxidation has received little attention. As far as we know, there is only one study, in which upon incubation of the LDL with Cu^{2+}, produced cholesteryl ester core aldehydes, such as 9-ONC and 5-OVC, as the major oxidized cholesteryl esters. The levels of 9-ONC and 5-OVC reached peaks at 12 h and significantly decreased thereafter (Fig. 2D). In addition, the reduction of the core aldehyde levels was accompanied by the formation of the free 7-ketocholesterol and 7-ketocholesterol ester core aldehydes (Fig. 3) and by the increase in the amounts of the apoB-bound cholesterol and 7-ketocholesterol (Fig. 4), suggesting that the cholesteryl ester core aldehydes were further converted to their 7-ketocholesterol and apoB-bound derivatives. On average, there were about 97 mol of cholesterol and 7-ketocholesterol attached per mol of apoB after 48 h of the copper-catalyzed oxidation. The overall decrease in the number of lysine residues averaged 250 mol/mol of apoB. Thus, 38.4% of the conjugated lysine amino groups appeared to be attributable to the cholesteryl ester core aldehydes. The remainder is presumably attributable to conjugation with phospholipid core aldehydes and with nonester fragments from polyunsaturated fatty acids, including MDA and 4-hydroxy-2-nonenal, and the many other aldehydic fragments that have been demonstrated (17).

To further ascertain the formation of 9-ONC covalently bound to protein in vivo, we obtained the murine monoclonal antibody, mAb 2A81, which clearly distinguished the 9-ONC-modified protein from the native protein (Fig. 6). Characterization of the antibody revealed that the monoclonal antibody was directed against protein-bound cholesteryl ester core aldehydes (Figs. 6 and 7). The in vivo detection of antigenic structures using mAb 2A81 was attempted in the tissue samples from the patients with atherosclerosis, which is considered to be a form of chronic inflammation resulting from the interaction between the modified lipoproteins, monocyte-derived macrophages, T cells, and the normal cellular elements of the arterial wall. We confirmed that atheromatous lesions indeed contained protein-bound cholesterols, colocalizing mainly with foamy macrophages (Fig. 8). It is known from the in vitro studies that all of the major cell types within the atherosclerotic lesions are capable of promoting the oxidation of LDL (1, 2). Therefore, the observed cell-associated staining patterns are probably attributable to the cellular oxidation of LDL by endothelial cells, macrophages, and smooth muscle cells. The resulting oxidized LDL may be taken up by cells and may be the ultimate source of the lipids that accumulate in the atherosclerotic lesions. In addition, the intracellular granular staining observed in the atherosclerotic lesions represents the presence of protein-bound cholesterols that had already been taken up by the macrophages and are present within the cell in cytoplasmic...
organelles (33). This leads to speculation that the modification by cholesteryl ester core aldehydes renders proteins relatively resistant to intracellular proteolytic degradation, resulting in the marked accumulation of epitopes in the macrophages. On the other hand, it has also been shown that core aldehydes produced from oxidized cholesteryl ester are less efficiently hydrolyzed by an acidic macrophage cholesteryl ester hydrolase than the native cholesteryl ester and that the site-specific release of core aldehydes in lysosomes could lead to the irreversible intralysosomal deposition of the lipid-protein complexes (13). This is compatible with lipid engulfment in lysosomes (34) and partial lysosomal enzyme inactivation after uptake of the oxidized LDL (35). Also, the predominant lysosomal localization of the epitopes specific for oxidized proteins (33), the lysosomal accumulation of ceroid (36), and the partially degraded apoB from oxidized LDL (37) indicate that the lysosome is the site where potential pathophysiological consequences of the core aldehydes could be manifested. In line with the suggestion of a relatively slow turnover, cholesteryl ester core aldehydes of different chain lengths have been found in the lipid extracts of atherosclerotic plaques (13, 14).

In summary, we analyzed the oxidized cholesteryl esters, such as 9-ONC and 5-OVC, generated during the peroxidation of LDL, and found that these core aldehydes were converted to their 7-ketocholesterol and apoB-bound derivatives. Moreover, we raised the monoclonal antibody directed to the protein-bound 9-ONC and obtained new murine monoclonal antibodies, mAb 2A81, that clearly distinguished the 9-ONC-modified protein from the native protein. The observation that the monoclonal antibody did not cross-react with proteins that had been treated with the reactive aldehydes but rather with the oxidized cholesteryl esters suggested that the antibody extensively recognized the protein-bound cholesteryl ester core aldehydes. Using mAb 2A81, we showed that immunoreactive materials were indeed formed not only in the oxidized LDL but also in the atherosclerotic lesions of the human aorta. These data suggested that the reaction between oxidized cholesteryl esters and primary amines of the proteins might represent a process common to the formation of carbonyl-modified proteins during aging and its related diseases.

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