Lipid Peroxidation Modification of Protein Generates Nε-(4-Oxononanoyl)lysine as a Pro-inflammatory Ligand

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4-Oxo-2(E)-nonenal (ONE), a peroxidation product of ω-6 polyunsaturated fatty acids, covalently reacts with lysine residues to generate a 4-ketoamide-type ONE-lysine adduct, Nε-(4-oxononanoyl)lysine (ONL). Using an ONL-coupled protein as the immunogen, we raised the monoclonal antibody (mAb) 9K3 directed to the ONL and conclusively demonstrated that the ONL was produced during the oxidative modification of a low density lipoprotein (LDL) in vitro. In addition, we observed that the ONL was present in atherosclerotic lesions, in which an intense immunoreactivity was mainly localized in the vascular endothelial cells and macrophage- and vascular smooth muscle cell-derived foam cells. Using liquid chromatography with online electrospray ionization tandem mass spectrometry, we also established a highly sensitive method for quantification of the ONL and confirmed that the ONL was indeed formed during the lipid peroxidation-mediated modification of protein in vitro and in vivo. To evaluate the biological implications for ONL formation, we examined the recognition of ONL by the scavenger receptor lectin-like oxidized LDL receptor-1 (LOX-1). Using CHO cells stably expressing LOX-1, we evaluated the ability of ONL to compete with the acetylated LDL and found that both the ONE-modified and ONL-coupled proteins inhibited the binding and uptake of the modified LDL. In addition, we demonstrated that the ONL-coupled protein was incorporated into differentiated THP-1 cells via LOX-1. Finally, we examined the effect of ONL on the expression of the inflammation-associated gene in THP-1 and observed that the ONL-coupled proteins significantly induced the expression of atherogenesis-related genes, such as the monocyte chemoattractant protein-1 and tumor necrosis factor-α, in a LOX-1-dependent manner. Thus, ONL was identified to be a potential endogenous ligand for LOX-1.

Atherosclerosis is a disorder of the lipid metabolism as well as a chronic inflammatory disease. Monocyte-derived macrophages play a prominent role in the formation and progression of atherosclerotic plaque, particularly after their transformation into foam cells. When activated by inflammatory stimuli, the macrophages synthesize and secrete various mediators, including cytokines, prothrombotic substances, and eicosanoids, which cause the clinical manifestations and acute clinical complications of atherosclerosis. Various lines of evidence have indicated that an important part of the pathogenesis of atherosclerosis is the modification of plasma low density lipoproteins (LDL) (1, 2). A large number of pro-inflammatory and pro-atherogenic properties have been ascribed to the oxidatively modified LDL (OxLDL) and their components (3). In particular, there is considerable evidence to support the role of oxidized fatty acids originating from the OxLDL as important signaling molecules in the context of the atherosclerotic lesion. Podrez et al. (4) have recently shown that the OxLDL components, such as oxidized phosphatidylcholines, serve as endogenous ligands for the scavenger receptor, CD36, facilitating macrophage cholesterol accumulation and foam cell formation. Nagy et al. (5) have shown that the oxidized fatty acids, such as the 9- and 13-hydroxyoctadecadienoic acids and 15-hydroxyeicosatetraenoic acid, regulate the macrophage gene expression through the ligand activation of the peroxisome proliferator-activated receptor γ. In addition, the lipid peroxidation-derived short chain aldehydes, such as acrolein and 4-hydroxy-2-nonenal, are considered important mediators of cell damage because of their ability to covalently modify biomolecules, which can disrupt important cellular functions and can cause mutations (6). These reactive aldehydes have been shown to modulate the NF-κB-dependent signaling pathways, which play an important role in gene regulation during inflammatory and immune responses (7). Furthermore, the addition of aldehydes to apolipoprotein B (apoB) in LDL has been strongly implicated in the mechanism by which LDL is converted into an atherogenic form that is taken up by macrophages, leading to the formation of foam cells (1, 2). The oxidized lipids generated during the oxidative modification of LDL are therefore likely to

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2 The abbreviations used are: OxLDL, oxidatively modified LDL; AcLDL, acetylated low density lipoprotein; SMA, α-smooth muscle actin; Bi-HSA, biotinylated human serum albumin; HSA, human serum albumin; KLH, keyhole limpet hemocyanin; OHE, 4-oxo-2-hexenal; ONE, 4-oxo-2-nonenal; ONL, Nε-(4-oxononanoyl)lysine; PDTC, pyrrolidine dithiocarbamate; PUFAs, polyunsaturated fatty acid; SR, scavenger receptor; DIO, 1,1′-diocadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate; CF, cyan fluorescent protein; ROS, reactive oxygen species; MRM, multiple reaction monitoring; DPI, diphenyldionidium.
be involved in the process of macrophage transformation into the foam cells during atherogenesis.

4-Oxo-2(E)-nonenal (ONE) is one of the major lipid peroxidation products and has been recently established to be formed by the free radical-initiated degradation of ω-6 PUFA s, such as linoleic acid and arachidonic acid (8, 9). ONE is also a potential inducer of cell death, including apoptosis in various cell lines (10–13), and a particularly potent lipid hydroperoxide-derived genotoxin (8), which reacts with DNA bases to form heptanone-etheno adducts (8, 14–18). Upon reaction with protein, ONE covalently modifies the arginine, cysteine, histidine, and lysine residues (19). Because of the instability of the adducts, the structures of the ONE-modified amino acid adducts have not been determined except for the ONE-arginine and ONE-cysteine adducts (20–22). However, Zhu and Sayre (23) have recently identified a novel 4-ketoamide-type ONE-lysine adduct, N\(^\text{\textsuperscript{\textalpha}}\)-(4-oxononanoyl)lysine (ONL), that arises from a reversibly formed ONE-lysine Schiff base (Fig. 1). They also demonstrated that the 4-ketoamide-type adduct is the most prominent long lived mono-lysine adduct formed by ONE (23).

In this study, to further evaluate the contribution of the protein modification by ONE in the pathogenesis of various diseases associated with oxidative stress, we raised a monoclonal antibody (mAb) directed to ONL and determined its formation in the foam cells during atherogenesis.

**Preparation of ONE**—ONE was synthesized by the oxidative opening of the 2-pentylfuran ring as described previously (22). Briefly, N-bromosuccinimide (Wako Pure Chemicals, Japan) and pyridine were sequentially added to 2-pentylfuran in THF/acetic acid/water (5:4:2) at ice bath temperature. The resulting mixture was stirred for 1 h at ice bath temperature and then stirred for 1 h at room temperature. The solvent was then removed by rotary evaporation, and the residue was chromatographed on a silica gel column (n-hexane/ethyl acetate = 3:1) (24).

**4-Oxononanoic Acid N-Hydroxysulfosuccinimide Ester**—A solution of 4-oxononanoic acid (172.3 mg, 1 mmol), prepared according to the published procedure (23), N-hydroxysuccinimide sodium salt (227.1 mg, 1.1 mmol) in 10 ml of dry N,N-dimethylformamide was stirred at 25 °C for 48 h under argon. The white precipitate was removed by filtration and washed with dry N,N-dimethylformamide. The filtrate was concentrated under vacuum to give a white residue. The residue was washed with ether (three times, 10 ml) and dried to give a white solid (353.7 mg, 95.2%), which was used without further purification.

**Preparation of 4-Oxononanoic Acid Keyhole Limpet Hemocyanin (KLH) Conjugate**—A standard KLH solution was prepared according to the published procedure (25), and the protein concentration (7.35 mg/ml) was determined by the bicinchoninic acid (BCA) assay using bovine serum albumin (BSA) as the standard. To the standard KLH solution (2.7 ml, 19.8 mg) was added 2.3 ml of doubly distilled water and 4-oxononanoic acid N-hydroxysuccinimide ester (16.3 mg, 0.044 mmol). The solution was vortexed at 25 °C for 4 h and then centrifuged, and the supernatant was dialyzed twice against 500 ml of a 2:1 (v/v) mixture of 100 mM NH\(_4\)Cl (pH 7.4) and methanol for 12 h and then twice against 500 ml of 50 mM sodium phosphate buffer (pH 7.4) for 12 h.

**Preparation of 4-Oxononanoic Acid BSA Conjugate**—BSA (5 mg) and 4-oxononanoic acid N-hydroxysuccinimide ester (11.4 mg, 0.031 mmol) were added into 5 ml of 50 mM sodium phosphate buffer (pH 7.4). The mixture was vortexed at 25 °C for 4 h and then dialyzed twice against 500 ml of a 2.1 (v/v) mixture of 100 mM NH\(_4\)Cl (pH 7.4) and methanol for 12 h and then twice against 500 ml of 50 mM sodium phosphate buffer (pH 7.4) for 12 h. The extent of lysine modification was determined with MALDI-TOF mass spectrometry by the procedure described previously (26).

**Preparation of Antibody**—Female BALB/c mice were immunized three times with 4-oxononanoic acid-coupled KLH. Spleen cells from the immunized mice were fused with P3U1 murine myeloma cells and cultured in hypoxanthine/aminopterin/thymidine selection medium. Culture supernatants of the hybridoma were screened using ELISA, employing pairs of wells of ELISA plates on which were absorbed 4-oxononanoic acid-coupled BSA as the antigen (5 μg of protein/well). After incubation with 100 μl of the hybridoma supernatants, and with intervening washes with phosphate-buffered saline (pH

**EXPERIMENTAL PROCEDURES**

**Materials**—The antibodies against LOX-1 (for immunoblot analysis), NF-κB (p65), and lamin A were obtained from R&D Systems (Minneapolis, MN), Santa Cruz Biotechnology, and Sigma, respectively. The anti-LOX-1 mouse monoclonal anti-body (for neutralizing experiments) was purchased from Abcam (Cambridge, UK). Trolox and pyrrolidine dithiocarbamate (PDTC) were from Sigma. Quercetin was obtained from Nakalai Tesque, Kyoto, Japan.
After discarding the supernatants and washing three times with PBS-T, 100 μl of antibody was added to each well and incubated for 1 h at 37 °C to block the unsaturated plastic surface. The plate was then washed three times with PBS-T. A 100-μl aliquot of the antigen solution was added to each well of a 96-well ELISA plate and incubated for 16 h at 4 °C. The antigen solution was then removed, and the plate was washed with Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Each well was incubated with 200 μl of 4% Block Ace (Yukijirushi, Japan) in TBS-T for 1 h at 37 °C to block the unsaturated plastic surface. The plate was then washed three times with PBS-T. A 100-μl aliquot of antibody was added to each well and incubated for 1 h at 37 °C. After discarding the supernatants and washing three times with PBS-T, 100 μl of a 5 × 10^5 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (MP Biomedicals) in PBS-T was added. After incubation for 1 h at 37 °C, the supernatant was discarded, and the plates were washed three times with PBS-T. Enzyme-linked antibody bound to the well was revealed by adding 100 μl/well of 0.03% hydrogen peroxide. The reaction was terminated by the addition of 50 μl of 2 M sulfuric acid, and absorbance at 492 nm was read on a micro-ELISA plate reader (Bio-Rad). For the competitive ELISA analysis, results were expressed as the ratio \( B/B_{	ext{w}} \), where \( B = \) (absorbance in the presence of competitor — background absorbance (no antibody)) and \( B_{	ext{w}} = \) (absorbance in the absence of competitor — background absorbance).

**SDS-PAGE and Immunoblot Analysis** — The proteins were separated by SDS-PAGE in the presence of 2-mercaptoethanol. The protein was stained with Coomassie Blue. The gel was transblotted onto a nitrocellulose membrane Hybond ECL (GE Healthcare), incubated with skim milk (50 mg/ml in TBS-T) for blocking, washed, and treated with the primary antibody. This procedure was followed by the addition of HRP-conjugated anti-mouse IgG and ECL reagents (GE Healthcare). The bands were visualized by LumiVision PRO 400EX (Aisin Seiki Co., Ltd., Aichi, Japan).

**Iron-catalyzed Oxidation of PUFAs** — HSA (1 mg/ml) was incubated with 20 mM PUFAs (arachidonic acid and linoleic acid) (Nu-Chek Prep, Inc., Elysian, MN) in the presence of 10 μM Fe^{2+} and 2 mM ascorbate in 50 mM sodium phosphate buffer (pH 7.2) at 37 °C. The reaction was terminated by adding diethylenetriaminepentaacetic acid (100 μM) and dibutylhydroxytoluene (1 mM).

**In Vitro Peroxidation of LDL** — LDL was separated from the plasma of healthy donor by ultracentrifugation as described in the literature (27) within a density cutoff of 1.019 to 1.063 g/ml and then dialyzed with cellulose membranes in PBS (pH 7.4) containing 100 μM EDTA. It was sterilized with a Mille-GV filter (Millipore) after dialysis. The protein concentration of LDL was measured using the bicinchoninic acid protein assay reagent (Pierce). LDL was dialyzed in PBS (pH 7.4) to remove EDTA. The oxidation of LDL (1 mg of protein/ml) by 5 μM Cu^{2+} was carried out at 37 °C under air in PBS (pH 7.4).

**Agarose Gel Electrophoresis** — Agarose gel electrophoresis was performed with the Titan Gel lipoprotein system (Helena Laboratories, Saitama, Japan) for lipoprotein samples and the Titan Gel high resolution protein system for protein samples. The samples were run on two separate gels. One gel was used for staining with Fat Red 7B, and the other was used for immunoblot analysis.

**Immunohistochemistry** — This investigation was carried out on aortic wall samples obtained during autopsy from patients with generalized arteriosclerosis. Each autopsy was performed at Tokyo Women’s Medical University after the family members granted informed consent according to the established guidelines. Each sample was fixed in 10% formalin, embedded in paraffin, and stored at room temperature or embedded in optimal cutting temperature compound (Sakura Finetech, Tokyo, Japan), frozen by the dry ice-ace tone method, and stored at −80 °C. Multiple 6-μm-thick sections were cut from these paraffin-embedded and frozen materials and used for histopathological and immunohistochemical examinations. Paraffin-embedded sections were deparaffinized and rehydrated, and frozen sections were air-dried, postfixed for 10 min at room temperature in 10% formalin, and rehydrated. These prepared sections were quenched for 10 min at 4 °C with 3% hydrogen peroxide for inhibiting endogenous peroxidase activity, rinsed in PBS, pretreated for 30 min at room temperature with 5% skim milk in PBS, and treated with the avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA). Sections were then incubated overnight at 4 °C with the primary antibodies such as the mAb 9K3 at a dilution of 1:200, polyclonal IgG against CD68 (Santa Cruz Biotechnology) at a dilution of 1:100, and polyclonal IgG against α-smooth muscle actin (SMA) (AbCam, Cambridge, MA) at a dilution of 1:500. Sections processed with omission of the primary antibodies or incubated with 5% skim milk in PBS served as negative reaction controls. Antibody binding was visualized by the avidin-biotin-immunoperoxidase complex method using the appropriate Vectastain ABC kit (Vector Laboratories). Immunohistochemical localization of the ONL was verified by comparison with consecutive sections stained with hematoxylin-eosin and immunostained for CD68 or SMA. 3,3′-Diaminobenzidine tetrahydrochloride was used as the chromogen and hematoxylin as the counterstain. Immunostained sections were observed with a light microscope (Olympus, Tokyo, Japan). In addition, the location of the ONL immunoreactivity in macrophages was strictly identified by the double immunofluorescence method on frozen sections. In brief, sections were postfixed for 10 min at 4 °C in 100% acetone, rehydrated, rinsed in PBS, pretreated for 10 min at room temperature with 5% skim milk in PBS, and incubated overnight at 4 °C with the mAb 9K3 and the anti-CD68 antibody or the anti-SMA antibody, simultaneously. Sections were then rinsed in PBS and incubated for 1 h at room temperature with...
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fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) at the same time. Double-immunostained sections were observed with a fluorescence microscope (Nikon, Tokyo, Japan). The appearance of yellowish signals at merging FITC (green) and Cy3 (red) was considered as the colocalization of the ONL and CD68 or SMA.

**LC-ESI-MS/MS Analysis**—Mass spectrometric analyses were performed using an ACQUITY TQD system (Waters) equipped with an ESI probe and interfaced with a UPLC system (Waters). The sample injection volumes of 10 μl each were separated on a Waters BEH C18 1.7-μm column (150 × 2.1 mm) at a flow rate of 0.3 ml/min. A discontinuous gradient was used by solvent A (H2O containing 0.1% formic acid) with solvent B (acetonitrile containing 0.1% formic acid) as follows: 5% B at 0 min, 5% B at 3 min, 40% B at 10 min, 95% B at 12 min. Mass spectrometric analyses were performed on line using ESI-MS/MS in the positive ion mode with MRM mode (cone potential 30 eV/collision energy 25 eV). The MRM transitions monitored were as follows: reduced [U-13C6,15N2]ONL, m/z 311.1 → 90.1; reduced ONL, m/z 303.2 → 84.1. The amount of reduced ONL adduct was quantified by the ratio of the peak area of the target adducts and of the reduced ONL-stable isotope. QuanLynx software (Waters) was used to create standard curve (supplemental Fig. S3) and to calculate the adduct concentrations.

For the LC-ESI-MS/MS analysis of the reduced ONL in vitro, the protein samples were transferred into a glass tube containing butylated hydroxytoluene in methanol (1 mm) and then reduced with 100 mM NaBH4 for 2 h. The samples were then treated with an equal volume of 20% trichloroacetic acid, mixed well, left on ice for 10 min, and then centrifuged (10,000 g, 4 °C). The supernatant was removed, and the pellet was washed with cold acetone (500 l) and hydrolyzed exhaustively by Pronase (Calbiochem) and leucine aminopeptidase (Sigma). Pronase (50 μl, 2 mg/ml) was added, and the samples were incubated at 37 °C for 24 h. The pH was then adjusted to 8.5, and MgCl2 was added to a final concentration of 5 mM. Acti-
vated leucine aminopeptidase solution (55 ml) was subse-
dually expressed CFP-tagged LOX-1 (29) were grown on cover-
slips. Each coverslip was inverted onto a glass slide with a spacer, and the cells were examined using a Leica DM IRE2 microscope (Leica Microsys-
tems, Wetzler, Germany) equipped with a ×100 NA 1.4 objec-
tive and a Cool SNAP-HG-digitalized cooled CCD camera (Roper Scientific, Trenton, NJ) driven by MetaMorph software (Universal Imaging, Downingtown, PA). The expression level of LOX-1 was examined by the CFP fluorescence intensity monitored using an E4 filter with excitation at 436 nm (7-nm bandpass) and a 470-nm long pass emission filter. Images were acquired at a 0.2-s exposure time. The DiD-AcLDL uptake was analyzed by the amount of the DiD-derived fluorescence intensity using a Y5 filter, with excitation using a 620-nm (50-nm bandpass) and 700-nm (75-nm bandpass) emission filter. The images were acquired at a 1-s exposure.

**Cell Culture**—Human monocyte cell line THP-1 cells were grown in RPMI 1640 medium (Nissui, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 0.2% NaHCO3, at 37 °C in an atmosphere of 95% air and 5% CO2. The cells were differentiated by incubation with 200 nM phorbol 12-myristate 13-acetate for 3 days, and then cultured with FBS-free RPMI 1640 medium for 24 h.

**Membrane Preparation and Ligand Blot Analysis**—Cells were suspended in 50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, 1 mM EDTA, and protease inhibitor mixture and homogenized with 20 strokes in a Potter-Elvehjem homoge-

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(400 μg/ml) at 4 °C for 12 h. This procedure was followed by the addition of HRP-conjugated streptavidin (GE Healthcare) and ECL reagents. The bands were visualized by LumiVision PRO 400EX.

Fluorescence Microscopy—Differentiated THP-1 cells were treated with AlexaFluor488-labeled HSA (HSA488) or ONL-coupled HSA488 (100 μg/ml) for 30 min and then fixed in PBS containing 3.8% paraformaldehyde for 30 min. The cells were then rinsed with PBS and covered with antifade solution (Invitrogen). Images of the cellular immunofluorescence were acquired using a confocal laser scanning microscope (LSM5 PASCAL; Zeiss).

Measurement of ROS—The differentiated THP-1 cells were precultured with serum-free RPMI 1640 medium for 24 h and then treated with or without DPI for 1 h. The cells were then incubated with 10 μM 2’,7’-dichlorofluorescin diacetate for 30 min at 37 °C. The dichlorofluorescin-loaded cells were exposed to native-HSA or ONL-coupled HSA for an additional 30 min at 37 °C and then washed with ice-cold PBS. The fluorescence was measured using the Typhoon 9400 (GE Healthcare).

RT-PCR Analysis—Total RNA was isolated from the cells using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol and spectrophotometrically quantified. The total RNAs (5 μg) were reverse-transcribed into cDNA and used for the RT-PCR analysis. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard. The PCR products were separated in a 1% agarose gel, and the positive signals were quantified by densitometry analysis after staining with ethidium bromide. The following primer pairs (Genset) were used: GAPDH, forward, 5'-AACCCATCACCATCTCGACGAC-3', and reverse, 5’-CACATCTCTTCAGTGCGACATG-3'; MCP-1, forward, 5’-CCCACGTCACCTGCTGTAT-3', and reverse, 5’-CAACACATCCCCAGGGTGA-3'; TNF-α, forward, 5’-AGCCATGTGTAGTCACAACC-3', and reverse, 5’-GGTGTGAGGGTGTCTGAGGA-3'.

RESULTS
Development of a Monoclonal Antibody Directed to ONL—To evaluate the contribution of the protein modification by ONE in the pathogenesis of various diseases associated with oxidative stress, we attempted to develop a mAb specific to ONL. The hapten used in this study was the ONL that was covalently attached to the carrier protein (KLH). As the screening antigen, the ONL-coupled BSA was also prepared. The extent of the modification was confirmed by a MALDI-TOF MS analysis (supplemental Fig. S1).

BALB/c mice immunized with the immunogens produced antibodies, as judged by ELISA techniques (data not shown). Hybridomas were prepared by the fusion of myeloma cells with the spleen cells of the immunized mice. Hybridomas, secreting antibodies against ONL, were detected by ELISA on plates coated with the ONL-coupled BSA or ONE-treated BSA. Among the 12 obtained clones, three clones labeled 9K1, 9K2, and 9K3 showed a relatively high reactivity to the coated antigens (data not shown). Finally, one clone, 9K3, that showed a good growth and distinctive recognition of the ONL-coupled BSA and ONE-modified BSA, was selected and used in this study.

Specificity of the Anti-ONL mAb 9K3—Despite an extensive screening of the hybridoma that produced monoclonal antibodies specific to ONL, it is still conceivable that the antibody recognizes the epitope originating from other lipid peroxidation products. Therefore, we examined the immunoreactivity of mAb 9K3 toward aldehyde-treated BSA by ELISA. The antibody specifically reacted with the ONL-coupled BSA and ONE-treated BSA but hardly reacted with the protein treated with 2-alkenals (C3 to C11) or 4-hydroxy-2-alkenals, suggesting that ONE was the only source of antigenic adducts generated in the protein (supplemental Fig. S2). In addition, the antibody did not cross-react either with the protein treated with 4-oxo-2-hexenal (OHE), the short chain analog of ONE, or with the BSA conjugate of N°-(4-oxohexanoyl)lysine (OHL), an OHE-derived 4-ketoamide adduct. Moreover, the immunoreactivity with the reaction products of ONE with N°-hippuryl-l-lysine was characterized. We observed that the reaction of ONE with N°-hippuryl-l-lysine provided a number of products (supplemental Fig. S3, middle panel). The ONL peak was identified by a retention time comparison of an authentic standard (supplemental Fig. S3, upper panel) and by an ESI-LS-MS analysis (m/z 462.2 [M+H]+). The ELISA analysis indeed showed that the binding of the ONE-modified protein to the mAb 9K3 was scarcely inhibited by N°-hippuryl-l-lysine but was significantly inhibited by N°-hippuryl-ONL (supplemental Fig. S3B). These results suggested that mAb 9K3 exclusively recognizes ONL.

Formation of ONL in the ONE-modified Proteins—To confirm the formation of ONL in protein treated with ONE, we performed SDS-PAGE and an immunoblot analysis with mAb 9K3. HSA (1 mg/ml) incubated with each concentration of ONE for 24 h was boiled with an SDS sample buffer and then separated by SDS-PAGE. As shown in Fig. 2A, the binding of ONE to HSA was suggested by a slight expanse and mobility shift in the protein bands on SDS-PAGE. Incubation of the protein with ONE resulted in the dose-dependent formation of ONL, which was accompanied by a dramatic increase in the immunoreactivity with mAb 9K3. In addition, HSA (1 mg/ml) was incubated with 1 mM ONE for each time point (0, 2, 4, 8, 12, 24, and 48 h) and was analyzed by SDS-PAGE and an immunoblot analysis. As shown in Fig. 2B, immunoreactivity was observed in the protein treated with ONE. Similar to the β-lactoglobulin cross-linking by ONE (26), HSA was cross-linked by ONE to form dimer and trimer (>150 kDa), which exhibited a strong immunoreactivity with mAb 9K3 as well (Fig. 2, A and B). Moreover, the time- and dose-dependent formation of ONL in the ONE-modified HSA was also confirmed by the ELISA analysis (Fig. 2, C and D).

Formation of ONL in the Protein Exposed to the Iron/Ascorbate-mediated Oxidation of PUFAs and in Oxidized LDL—To examine whether the peroxidation of the PUFAs could generate antigenic structures in a protein, we incubated the arachidonic acid and linoleic acid with an iron/ascorbate-mediated free rad-
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A, HSA (1.0 mg/ml) was incubated with the indicated concentrations of ONE in 50 mM sodium phosphate buffer (pH 7.4) at 37 °C for 24 h. Left panel, SDS-PAGE and Coomassie Brilliant Blue (CBB) staining; right panel, immunoblot analysis with mAb 9K3. B, HSA (1.0 mg/ml) was incubated with 1 mM ONE in 50 mM sodium phosphate buffer (pH 7.4) at 37 °C for the indicated periods of time. Left panel, SDS-PAGE and Coomassie Brilliant Blue staining; right panel, immunoblot analysis with mAb 9K3. C and D, ELISA analysis using mAb 9K3. The results shown are means ± S.D. of three independent experiments.

FIGURE 2. Dose- and time-dependent formation of the ONL. A and B, formation of the ONL in the protein exposed to iron/ascorbate oxidation of PUFAs. HSA (1 mg/ml) was incubated with 20 mM PUFAs in the presence of 10 μM iron and 2 mM ascorbate in 50 mM sodium phosphate buffer (pH 7.2) at 37 °C. A, immunoreactivity of mAb 9K3 to HSA exposed to iron/ascorbate oxidation of PUFAs. The immunoreactivity of mAb 9K3 was determined by a direct ELISA using HSA exposed to iron/ascorbate oxidation of PUFAs as the absorbed antigen. The results shown are means ± S.D. of three independent experiments. Closed circles, linoleic acid; open circles, arachidonic acid. B, SDS-PAGE/immunoblot analysis of the HSA exposed to iron/ascorbate oxidation of arachidonic acid. The left panel shows the results of SDS-PAGE and Coomassie Brilliant Blue (CBB) staining. The right panel shows the results of immunoblot analysis with mAb 9K3. C and D, formation of the ONL in Cu²⁺-oxidized LDL. LDL (1 mg) was incubated with 5 μM Cu²⁺ in 1 ml of 50 mM sodium phosphate buffer (pH 7.2) at 37 °C. C, agarose gel electrophoresis/immunoblot analysis of OxLDL. Upper panel, Fat Red 7B staining; lower panel, immunoblot analysis with mAb 9K3. Lane ONE-LDL shows the level of the formation of the ONL in the LDL (1 mg/ml) treated with ONE (1 mM) for 24 h. D, ELISA analysis of OxLDL using mAb 9K3. The results shown are means ± S.D. of three independent experiments.

As shown in Fig. 3A, the iron/ascorbate-mediated oxidation of arachidonic acid and linoleic acid in the presence of HSA resulted in a time-dependent increase in the antigenicity of the protein. In addition, the formation of the ONL in the modified protein was also confirmed by an immunoblot analysis using mAb 9K3 (Fig. 3B). This result is consistent with the previous report showing that ONL adduct was detected by mass spectrometry in β-lactoglobulin exposed to iron/ascorbate-mediated oxidation of linoleic acid (30). HSA treated with the iron/ascorbate/linoleic acid system was shown to form HSA oligomers (>150 kDa), which was presumably due to the protein cross-linking by lipid peroxidation-derived aldehydes. Thus, ONL was confirmed to be formed during the reaction between the protein and ONE generated during the lipid peroxidation.

An important part of the pathogenesis of atherosclerosis has been implicated by the oxidative modification of LDL (1, 2). The modification of LDL involves the oxidation of PUFAs included in the LDL particle along with the appearance of lipid peroxidation products, such as electrophilic aldehydes (31, 32). It has been shown that these aldehydes form covalent bonds with the ε-amino groups of the lysine residues in apoB-100, leading to a decrease in the net negative charge of the LDL particle and to an increase in its electrophoretic mobility (33). To determine whether the antigenic structures recognized by mAb 9K3 are formed in OxLDL, LDL treated with 5 μM Cu²⁺ was subjected to an agarose gel electrophoresis followed by immunoblot analysis with mAb 9K3. The incubation of LDL with Cu²⁺ led to oxidation of the LDL as assessed by the formation of TBARS (data not shown). After separation by agarose gel electrophoresis, the native form of the LDL appeared as a single protein band that was readily visualized by Fat Red 7B staining (Fig. 3C, upper panel); however, the LDL incubated with 5 μM Cu²⁺ exhibited an enhanced anodic mobility compared with the native LDL, indicating an increased negative charge on the molecule, probably caused by the modification of
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lesional macrophages. In addition, the immunoreactivity was also colocalized in the vascular smooth muscle cell-derived foam cells identified by the specific marker α-smooth muscle actin (Fig. 4, G–I). Thus, the detection of ONL in the atherosclerotic plaques supports the notion that the reaction between ONE and the primary amines might represent a process common to the LDL modification during aging and its related diseases.

LC-ESI-MS/MS Analysis of ONL—To obtain direct evidence for the formation of the antigenic ONL in vivo, we attempted to establish a method for the detection of ONL using LC-ESI-MS/MS. Because the ONL has the same molecular weight as ONE-Lys Michael adduct, we sought to detect the ONL adduct as the NaBH₄-reduced form (Fig. 5A). Zhu and Sayre (23) have indeed shown that, upon NaBH₄ reduction, its molecular mass increased by 2 Da, whereas reduction of the ONE-Lys Michael adduct resulted in an increase of 4 Da. Collision-induced dissociation of the reduced ONL produced relevant product ions at m/z 84, 130, 147, 222, and 285 (Fig. 5B). These ions were applied to the structure shown in Fig. 5A. The product ions at m/z 84, 130, and 147 were confirmed to be the product ions of a lysine moiety, and the ion at m/z 285 originated from the reduced ONL. Although the product ion at m/z 222 was not assigned, the same ion peak was also observed in the previous report (23).

Fig. 5C demonstrates the LC-ESI-MS/MS analysis of the reduced ONL (0–2 pmol) in the positive ion mode using MRM. The characteristic product ion (m/z 303.2 → 84.1), allowing detection of reduced ONL. Chromatograms for the internal standard are shown in supplemental Figs. S4 and S5. Using the LC-ESI-MS/MS technique, we attempted to detect ONL in the ONE-modified HSA. Because general methods for acid hydrolysis of protein (e.g. 6 N HCl at 105 °C for 24 h) may result in cleavage of amide linkage in ONL, proteins were enzymatically hydrolyzed with Pronase and leucine aminopeptidase. As shown in Fig. 5, D and E, no adducts were detected in the native HSA, whereas treatment of HSA with 1 mM ONE in 50 mM sodium phosphate buffer (pH 7.2) for 24 h at 37 °C gave 370 nmol of the ONL per mol of protein.

To examine the involvement of the lipid peroxidation during the formation of ONL, we sought to detect reduced ONL in the protein exposed to the iron/ascorbate-mediated oxidation of linoleic acid using LC-ESI-MS/MS. As shown in Fig. 6, A and B, we confirmed a time-dependent formation of ONL. After 24 h of incubation, the amount of ONL was ~44 mmol/mol of HSA. In addition, we confirmed the formation of ONL in the Cu²⁺-oxidized LDL. The chromatograms of the product ion scan (precursor as m/z 303.2) (supplemental Fig. S7) of the reduced ONL fraction coincided with that of the standard reduced ONL (Fig. 5B), confirming the presence of ONL in the OxLDL. As shown in Fig. 6, C and D, the Cu²⁺-induced peroxidation of LDL dramatically enhanced the formation of ONL. The oxidation of LDL with Cu²⁺ for 24 h gave ~203 mmol of the ONL per mol of apob.

Based on the immunohistochemical findings that the ONL is present in the human atherosclerotic lesions (Fig. 4), we tried to quantify the ONL adduct in the athero-prone regions of the apoE-deficient mice, a model of atherosclerosis. Nor-
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A

B

C

D

E

m/z 303.2>84.1

Relative intensity (%)

Relative intensity (%)

Retention time (min)

Retention time (min)

Relative intensity (%)

Relative intensity (%)

Relative intensity (%)

Reduced ONL (mol/mol HSA)

ONE (mM)
mal and athero-prone regions from the apoE-deficient mice were analyzed for ONL using LC-ESI-MS/MS. The product ion spectrum (precursor as m/z 303.2) of the reduced ONL fraction was identical to that of authentic standard (Fig. 5B and supplemental Fig. S8), suggesting the presence of ONL in the athero-prone regions. As shown in Fig. 7, A and B, the ONL levels in the athero-prone regions were higher than those in the normal regions. However, the levels of the adduct were about 60 fmol/mg tissue protein. They were extremely low in comparison with those detected in the OxLDL in vitro.

Recognition of ONL by LOX-1—The finding that the ONL adduct is accumulated in the macrophage-derived foam cells in atherosclerotic lesions (Fig. 4) prompted us to hypothesize that the ONL adduct could be recognized as a ligand by a membrane protein such as a scavenger receptor in the macrophages. Based on previous findings that the scavenger receptor LOX-1 might be involved in the processing of aldehyde-modified proteins (34, 35) and that, among the aldehyde-treated proteins, the ONE-modified protein can be very efficiently incorporated into the LOX-1-overexpressing cells,3 we sought to determine whether LOX-1 recognizes the ONL adduct. AcLDL was used as an alternative ligand to the OxLDL, which shows a comparable affinity to LOX-1 (36) to avoid ambiguous effects from variations in the extent of the oxidation of LDL (37, 38). In this assay, the amount of incorporated DiD-labeled AcLDL (DiD-AcLDL) directly reflected the binding activity of LOX-1, which was confirmed by an independent analysis as described previously (39). The rate of DiD-AcLDL taken up by CHO stably expressing CFP/LOX-1 was assessed by a fluorescence microscope equipped with a cooled CCD camera. The stable cell line expressed a moderate amount of LOX-1, and fluorescence derived from DiD-AcLDL was observed (Fig. 8A). The effect of the ONE-modified HSA upon DiD-AcLDL uptake through LOX-1 was examined in the competition assay to evaluate the LOX-1 recognition of the modified proteins. As shown in Fig. 8C, the ONE-modified HSA significantly inhibited the uptake of DiD-AcLDL, whereas the native HSA had little inhibitory effect (Fig. 8B). In addition, we also observed similar inhibitory effects of the ONL-coupled HSA on DiD-AcLDL incorporation (Fig. 8D and supplemental Fig. S9).

We also tried to examine the binding of ONL to LOX-1 of differentiated human monocyte/macrophage THP-1 cells by a ligand blot analysis. The membrane proteins extracted from differentiated THP-1 cells were separated by SDS-PAGE under nonreducing conditions, transferred to a PVDF membrane, and used for the ligand blot analysis with the ONL-coupled and ONE-treated biotinylated HSA (Bt-HSA) as ligands. These ligands showed a predominant binding to a protein migrating to the same molecular mass (48 kDa) of LOX-1 as determined by immunoblot analysis, whereas the control Bt-HSA did not (Fig. 9). The binding of ONL-coupled and ONE-treated Bt-HSA to the 48-kDa protein was significantly inhibited by the addition of the excess unlabeled ONL-coupled HSA (Fig. 9).

In addition, using the fluorescence-labeled HSA, we examined the binding/uptake of the ONL-coupled protein in differentiated THP-1. As shown in Fig. 10, G–J, the ONL-coupled HSA was significantly incorporated into cells, whereas the control HSA was not (Fig. 10, D–F). Moreover, we also confirmed the inhibitory effect of the anti-LOX-1-neutralizing antibody on the binding/uptake of the ONL-coupled protein in THP-1 cells (Fig. 10, M–O). These results suggest that the scavenger receptor LOX-1 recognizes the ONL as a ligand.

Induction of Chemokine/Cytokine Expression by ONE-modified Protein in Differentiated THP-1 Cells—LOX-1 activation has been suggested to induce several intracellular signaling pathways, including the activation of NADPH oxidase on the cell membrane, resulting in the activation of the transcription factor NF-κB and subsequent downstream inflammatory responses (40). Hence, we sought to examine the effect of ONL on the expression of the inflammation-associated gene in differentiated THP-1 cells. After treating the THP-1 cells with the ONL-coupled HSA or native HSA for 24 h, we isolated the total RNA and subjected it to RT-PCR analysis. As shown in Fig. 11A, the expressions of the monocyte chemoattractant protein 1 (MCP-1) and tumor necrosis factor α (TNF-α) were significantly up-regulated by the ONL-coupled HSA. Similar results were obtained in the cells treated with the ONE-modified HSA (Fig. 11B). In addition, to confirm the contribution of LOX-1 to the cytokine/chemokine expression, we examined the effect of the anti-LOX-1-neutralizing antibody on the macrophage activation. As shown in Fig. 11C, the induction of MCP-1 and TNF-α expression was significantly hampered by the introduction of the anti-LOX-1-neutralizing antibody into the medium, whereas the normal mouse IgG had little effect. These data suggested that the ONL-induced expression of the inflammatory-related gene is LOX-1-dependent.

Next, we investigated the contribution of NADPH oxidase to the induction of the inflammatory gene. As shown in Fig. 12A, the NADPH oxidase inhibitor DPI significantly inhibited the ONL adduct-induced expression of MCP-1 and TNF-α. We also confirmed that the production of the intracellular ROS was induced by the ONL-coupled protein, and its production was suppressed by the pretreatment of DPI (Fig. 12B). Moreover, the expression of MCP1 and TNF-α induced by the ONL-coupled protein was inhibited by the antioxidants, Trolox and quercetin (Fig. 12C).

Finally, to determine whether NF-κB indeed contributes to the ONL-induced expression of MCP-1 and TNF-α, we examined the nuclear localization of NF-κB p65 in the ONL-coupled HSA-treated THP-1 cells. As shown in Fig. 12D, the ONL-cou-
pled and ONE-modified HSA enhanced the nuclear accumulation of NF-κB p65. In addition, the pretreatment and continued presence of PDTC, an NF-κB inhibitor, partially reduced the ONL-coupled HSA-induced expression of MCP-1 and TNF-α (Fig. 12E). These results suggested that NF-κB is involved in the inflammatory response to the ONL.
Zhu and Sayre (23) have shown that the ONE-amine adduct that corresponds to the expected mass of the Michael adduct was actually an isomeric 4-ketoamide utilizing phenethylamine as a model compound. Using the Lys derivative, they also have determined the relative yield of the various ONE adducts as a function of time and found that the ONL adduct was shown to survive at the expense of the short lived Schiff base and Michael adducts. Although the ONL represents the major long lived noncross-linked ONE modification on the Lys residues (23), previous studies of the ONE that missed this ONL adduct either mainly focused on the early and/or reversibly formed ONE-derived adducts (11) or on adducts that could be readily detected in preparative isolation work by characteristic UV-visible spectral and NMR spectrometric signatures (21, 41, 42). In addition to ONE, 9,12-dioxo-10(E)-dodecaenoic acid, an ONE-like lipid peroxidation product that contains the carboxyl terminus, covalently reacts with the Lys residues to generate the same 4-ketoamide-type adduct (23). Williams et al. (43) also showed that 9,12-dioxo-10(E)-dodecaenoic acid 4-ketoamide was the major product of cytochrome c exposed to 13-hy-
droperoxyoctadecadienoic acid, an initial product of linoleic acid peroxidation, in the presence of ascorbate. As for the mechanism of formation of the 4-ketoamide-type adduct, it has been noted that the most direct route to the 4-ketoamide-type adduct is simply what should be the facile tautomerization of the carbinolamine precursor to the 4-oxo-2-enal-derived Schiff base (Fig. 1) (23).

In this study, using the chemically synthesized hapten (ONL)-protein conjugate as the immunogen, we successfully raised the mAb 9K3. This antibody turned out to be extremely specific for the ONE-modified proteins and hardly reacted with the proteins treated with the ONE analogs, such as 4-hydroxy-2-nonenal, 2-nonenal and OHE (supplemental Fig. S2). These observations and the observation that mAb 9K3 did not cross-react with the protein conjugated with OHL, an OHE-derived 4-ketoamide adduct, suggested that both the ketone and pentyl moieties of the ONL adduct may be critical for the antibody binding (supplemental Fig. S2). The mAb 9K3 was found to be capable of detecting ONL in the protein exposed to the iron/ascorbate-mediated oxidation of the PUFAs and in the OxLDL (Fig. 2), suggesting the usefulness of this antibody for further investigations aimed at elucidation of the relative contribution of ONE to the accumulation of oxidatively modified proteins in vivo. The detection of ONL 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 the protein-bound ONE, mainly colocalizing with foamy macrophages (Fig. 4). 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 likely attributable to the cellular oxidation of LDL by macrophages, smooth muscle cells, and endothelial cells. The resulting OxLDL 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 ONL that had already been taken up by the macrophages and are present within the cell in cytoplasmic organelles. This leads to the speculation that the modification by ONE renders proteins relatively resistant to intracellular proteolytic degradation, resulting in the marked accumulation of epitopes in the macrophages.
As we mentioned above, immunological detection using antibody is a potent tool that can be used to evaluate the presence of a desired target and its subcellular localization. However, because of the significant number of interfering substances in biological samples, antibody-based methods are insufficient for quantification. On the other hand, LC-MS/MS is a powerful technique for quantitative analysis when evaluated on the basis of speed, specificity, reliability, and sensitivity. In this study, we successfully established a method for the quantification of ONL using LC-ESI-MS/MS (Figs. 5 and 6) and accurately estimated the biological levels of the ONL in atheropane regions of apoE-deficient mice (Fig. 7). Both immunological (Fig. 4) and mass spectrometric (Fig. 7) detections provide strong evidence for the presence of ONL in vivo.

One of the most important functions of macrophages is the processing of altered self-components. In atherosclerosis, differentiated macrophages express a large group of pattern recognition receptors, such as scavenger receptors (SRs), involved in the recognition and phagocytic clearance. This encompasses the class A SRs, consisting of SR-A I/II/III, macrophage receptor with collagenous structure, the class B SRs, consisting of CD36, SR-B I/II, and the rest of the class, including CD68, lectin-like oxidized LDL receptor-1 (LOX-1), SRs expressed by endothelial cells I/II, and SR-PSOX (reviewed in Ref. 44). These receptors may recognize distinct epitopes on the modified LDL and participate in internalization of the LDL particles based on in vitro observations (45). Among them, LOX-1 was identified as a potential binding protein for the ONL adduct in this study (Figs. 8–10). LOX-1 is a type II membrane glycoprotein belonging to the C-type lectin family molecules, which can act as a cell surface endocytosis receptor for the atherogenic OxLDL (46).

The expression of LOX-1 is not constitutive but could be induced by proinflammatory stimulators, oxidative stress, and mechanical stimuli (47, 48). The up-regulated LOX-1 is involved in the activation of the endothelial cells, transformation of smooth muscle cells, and accumulation of lipids in macrophages, resulting in cell injury that facilitates the development of atherosclerosis (49). The relationship between LOX-1 and atherosclerosis has been reported as follows: (i) the LOX-1 expression is detected in the atherosclerotic lesions of animals and humans (50, 51); (ii) the LOX-1 expression is enhanced by atherosclerosis-related risk factors such as proinflammatory cytokines (52, 53); (iii) LOX-1 functions as a cell adhesion molecule mediating the platelet-endothelium interaction and is involved in the endotoxin-induced inflammation that may initiate and promote atherosclerosis (54, 55); and (iv) some antiatherosclerotic drugs, such as HMG-CoA reductase inhibitors statins, inhibit the up-regulation of LOX-1 and the uptake of OxLDL (56, 57). In addition, the overexpression of LOX-1 in apoE−/− mice results in a widespread vascular inflammation in atherosclerotic lesions and also in normal arteries (58). We demonstrated that the ONL-coupled HSA and ONE-treated HSA significantly inhibited the LOX-1-mediated uptake of AcLDL (Fig. 8). In addition, we also found that the ONL-coupled HSA was incorporated into differentiated THP-1 cells in a LOX-1-dependent manner (Fig. 10). These data suggested that ONL, as a ligand for LOX-1, might be involved in the atherosclerosis process.

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should also be careful when we claim that ONL is a ligand of LOX-1, because most of the experiments in this study were employed using synthetic ligands, such as ONE-modified and ONL-coupled HSA. In fact, endogenous proteins could have quite different lipid hydroperoxide-derived modifications, including those from ONE-like bifunctional electrophiles arising from the carboxyl terminus (43) and those arising from interactions of ONE with proximal histidine and lysine residues (21).

Despite the role in OxLDL endocytosis, the ligation of LOX-1 by OxLDL activates the inflammatory signal transduction pathway. Most of the findings about the LOX-1-mediated signal transduction pathway came from the activation or inhibition of LOX-1 by OxLDL and anti-LOX-1-neutralizing antibody. Using bovine aortic endothelial cells and LOX-1-transfected CHO-K1 cells, Cominacini et al. (40, 59) reported that formation of the OxLDL-LOX-1 complex induces a sharp increase in the intracellular ROS production via NADPH oxidase, whereas the native LDL and malondialdehyde-treated LDL had little effect. In addition, the binding of OxLDL to LOX-1 results in the activation of the transcription factor NF-κB as determined by an electrophoretic mobility shift assay (40). The LOX-1-mediated NF-κB activation by OxLDL is crucial for increasing the expressions of the MCP-1, endothelin-1, and adhesion molecules in macrophages and endothelial cells (60, 61). In this study, the treatment of the differentiated THP-1 cells with the ONL-coupled HSA indeed induced the production of ROS (Fig. 12B), and activated the NF-κB (Fig. 12D). In addition, the cytokine/chemokine induction was attenuated by the LOX-1-neutralizing antibody (Fig. 11C). These data suggest that the ONL adducts might participate in the LOX-1-mediated recognition of OxLDL and macrophage activation. Meanwhile, the possibility still exists for other scavenger receptors to be involved in the recognition of the ONE-modified proteins. We indeed observed that the anti-LOX-1-neutralizing antibody could not completely inhibit the cytokine expression and binding/uptake of the ONL-coupled protein in THP-1 cells (Figs. 10 and 11C).

The level of ONL in the ONE-modified protein that shows significant LOX-1 ligand activity was about 370 mmol/mol protein (Fig. 5), whereas the level of ONL in the oxidized LDL was about 200 mmol of ONL per mol of apoB (Fig. 6). These data suggest that the ONL adduct generated during lipid peroxidation reactions in the OxLDL could, at least in part, act as a LOX-1-dependent pro-inflammatory ligand. We could also detected ONL in the athero-prone regions of apoE-deficient mice in vivo; however, only femtomole levels of adducts were detected in vivo, yet millimole per mol levels of adducts on LDL are required for acquisition of significant binding to LOX-1. Thus, it remains to be elucidated whether the ONL adduct has physiological and/or pathophysiological significance as an endogenous ligand for LOX-1 in vivo. Further investigations are therefore necessary to establish the relationship between the level of modification needed to convert proteins into ligands and the levels found in vivo.

In summary, we have obtained a murine monoclonal antibody, mAb 9K3, which clearly distinguished the ONE-modified protein and OxLDL from the native protein and LDL. Using this antibody, it was shown that atherosclerotic lesions contained antigenic materials in the granular cytoplasmic elements of the foam cells. In addition, using LC-ESI-MS/MS, the biological levels of the ONL in vitro and in vivo were accurately estimated. Moreover, we identified the LOX-1 as a scavenger receptor that recognizes ONL and found that ONL could induce the inflammatory responses in a LOX-1-dependent manner. The present results not only show that the protein-bound ONE is potentially one of the most important markers of oxidative stress, but it also provide clues to the pathophysiological mechanisms of oxidative stress-related diseases such as atherosclerosis.

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