Detection of lipofuscin-like fluorophore in oxidized human low-density lipoprotein

4-Hydroxy-2-nonenal as a potential source of fluorescent chromophore

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Abstract It has recently been shown that the lipid peroxidation product 4-hydroxy-2-nonenal (HNE) forms a fluorescent hydroxyiminodihydropyrrole derivative with the ε-amino group of lysine residue. In this study, we raised a monoclonal antibody (mAb2C12) directed to the fluorophore–protein conjugate and found that the antibody was specific to the chromophore structure of the compound. Immunohistochemical analysis of atherosclerotic lesions from the human aorta showed that the fluorophore was indeed present in the lesions, in which intense positivity was primarily associated with macrophage-derived foam cells and thickening of the neointima of the arterial walls. Antigenic materials were also detected in the oxidatively modified low-density lipoprotein (LDL) with Cu^{2+} and in the oxidatively modified bovine serum albumin with an iron/linoleic acid autoxidation system, indicating that the HNE, which originated from the peroxidation of polyunsaturated fatty acids, could be a potential source of the fluorescent chromophore in oxidized LDL.

Key words: Lipid peroxidation; 4-Hydroxy-2-nonenal; Fluorescent chromophore; Low-density lipoprotein; Atherosclerosis

1. Introduction

It is known that one of the most remarkable features of low-density lipoprotein (LDL) is fluorescence (around $\lambda_{ex} = 360$ nm, $\lambda_{em} = 430$ nm). Due to its high sensitivity, this fluorescence is widely used for monitoring the oxidative modification of LDL. It has been suggested that fluorophores formed in oxidized LDL are generated by covalent binding of lipid peroxidation products to the lysine ε-amino groups of the LDL apolipoprotein B-100 (apoB) [1]. This lysine modification is also believed to be responsible for the recognition of the oxidized LDL by the scavenger receptor on macrophages. Lipid peroxidation of LDL produces various aldehydes, one of the most reactive being 4-hydroxy-2-nonenal (HNE). HNE generated during the oxidative modification of LDL has been shown to form stable non-fluorescent Michael addition-type products with the histidine and lysine residues of LDL apoB (Fig. 1A) [2–4]. An immunochemical study has demonstrated that these non-fluorescent adducts are indeed present in the LDL oxidatively modified by Cu^{2+} or cultured endothelial cells [2] and in human atherosclerotic lesions [4]. Whereas, it is striking to note that the treatment of LDL with HNE generates the same fluorescence properties as seen in oxidized LDL [5]. This finding suggests that HNE could be a major contributor to the fluorescence in oxidized LDL. We have recently identified the major lipofuscin-like fluorophore derived from HNE and lysine to be the hydroxyiminodihydropyrrole (HIDP) derivative (Fig. 1B) [6] and found that the fluorescent properties of this pigment are similar to those of the oxidized LDL. Furthermore, the formation of this fluorophore has been determined in the protein treated with HNE [7].

To understand the mechanism of the oxidative modification of lipoproteins in vivo, we initiated studies on the identification of covalently modified amino acids generated during in vitro incubation of the proteins with lipid peroxidation products. In the present study, we raised a monoclonal antibody directed to the fluorophore and showed evidence that the fluorophore is indeed present in the atherosclerotic lesions of the human aorta. In addition, we identified HNE to be a potential source of fluorescent pigment generated during the in vitro peroxidation of human plasma lipoproteins.

2. Materials and methods

2.1. Materials

HNE and 4-oxo-2-nonenal (ONE) were prepared according to the procedure of Grée et al. [8]. HIDP (**1**) was prepared from N^{a}-*t*-Boc-L-lysine and ONE according to the procedure of Xu et al. [9]. Preparative high-performance liquid chromatography (HPLC) was carried out on a C18 reversed phase column (5 μm particle size, 8.0×250 mm) at a flow rate of 2.0 ml/min. Products were monitored with a fluores-

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Abbreviations: HIDP, hydroxyiminodihydropyrrole; HNE, 4-hydroxy-2-nonenal; ONE, 4-oxo-2-nonenal; LDL, low-density lipoprotein; apoB, apolipoprotein B-100; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin

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cence detector at ex/em 360/430 nm. Isolated fluorescent products were stored as a water solution containing a small amount of CH_3CO_2H.

2.2. Preparation of a maleimide-activated HIDP derivative

The HIDP derivative **1** (1.0 mg) in 0.25 ml of 0.1 M phosphate buffer (pH 7.4) was mixed with 10 mg of *N*-(6-maleimidocaproyloxy)succinimide in 0.25 ml of dimethyl sulfoxide and left overnight at room temperature. A HPLC purification eluting with H_2O–CH_3CO_2H (100:1) containing 0–70% CH_3OH, linear gradient over 40 min, afforded 1.53 mg of 1-(N^{α}-(6-maleimidocaproyl)-L-lysyl)-2-hydroxy-2-pentyl-3-(N^{α}-(6-maleimidocaproyl)-L-lysylimino)-1,2-dihydropyrrole (**2**) as a mixture of two diastereomers.

2.3. Preparation of an HIDP-carrier protein

A sulfhydryl-containing keyhole limpet hemocyanin (KLH–SH) was prepared by a modification of the method of Hartley et al. [10]. To conjugate **2** to KLH–SH, 1.0 ml of phosphate-buffered saline (PBS) solution containing 1.0 mg of KLH–SH and 0.95 mg of **2** was allowed to stand overnight at room temperature. Due to the precipitation of the conjugate (HIDP–KLH), it was directly subjected to immunization in mice without purification. The HIDP-conjugated bovine serum albumin (HIDP–BSA) was prepared by the same method as HIDP–KLH.

2.4. Preparation of hybridomas

BALB/c mice were injected with 17 μg of the conjugate (HIDP–KLH). At 2 week intervals, they were boosted with 10 mg of the conjugate. Three days after the final boost, spleen cells from one of them were fused with P3/U1 murine myeloma cells. Aliquots of the hybridoma supernatants were tested for the presence of anti-HNE-treated BSA by enzyme-linked immunosorbent assay (ELISA). The ELISA was performed as described previously [11].

2.5. In vitro modification of LDL

LDL was separated from the plasma of a healthy donor by ultracentrifugation within a density cut-off 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 Mille-GV filter (Millipore) after dialysis. The protein concentration of the LDL was measured using the bicinchoninic acid protein assay reagent (Pierce).

2.6. In vitro modification of BSA

The iron-catalyzed oxidation of linoleic acid in the presence of BSA was performed by incubating BSA (1 mg/ml) with 5 mM linoleic acid in the presence of either 10 μM Fe^{2+}/1 mM ascorbate or 0.1 mM $FeCl_3$/0.1 mM EDTA/1 mM ascorbate in 0.5 ml of 0.1 ml of 0.1 M sodium phosphate buffer (pH 7.4) under atmospheric oxygen. The incubation was carried out at 37°C for 24 h.

2.7. 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 Fat Red 7B; the other was transblotted to nitrocellulose membranes, incubated with Block Ace (40 mg/ml) for blocking, washed and treated with the primary antibody. This procedure was followed by the addition of horseradish peroxidase conjugated to a goat anti-mouse IgG F(ab′)2 fragment and ECL reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). The bands were visualized by exposure of the membranes to autoradiography film.

2.8. Human samples of aorta

Specimens of human abdominal aorta were obtained from the autopsy cases of Kyoto University Hospital. Three cases (a 1-month-old, one 2-year-old and two 65-year-olds) were used. Whole aortic layers of grossly normal areas and/or areas containing fibrofatty atherosclerotic lesions were obtained within 6 h after death. The tissue was immediately fixed with Bouin's solution [12] overnight, sequentially immersed in 50% ethanol and 70% ethanol for 24 h, to remove picric acid, dehydrated, embedded in paraffin, sectioned at 3.5 μm and mounted on glass slides either for hematoxylin and eosin staining or immunohistochemistry.

2.9. Immunohistochemistry

The avidin–biotin complex (ABC) method with alkaline phosphatase was used as previously described [13]. Briefly, after the fixation procedures, normal rabbit serum (Dako Japan Co., Kyoto, Japan; diluted to 1:75) for the inhibition of non-specific binding of the secondary antibody, mAb2C12, biotin-labeled rabbit anti-mouse IgG serum (Dako; diluted 1:300) and ABC (Vector; diluted 1:100) were sequentially used. Procedures using PBS or the IgG fraction of the normal mouse serum instead of mAb2C12 showed no or negligible positivity. Procedures using PBS or the IgG fraction of normal mouse serum instead of the antibody showed no or negligible positivity.

3. Results and discussion

3.1. A monoclonal antibody directed to the HIDP fluorophore

To verify the formation of the HNE-derived HIDP fluorophore in oxidatively modified LDL, a monoclonal antibody was raised against the IDP–KLH conjugate which was prepared from the maleimide-activated HIDP derivative **2** and KLH–SH (Fig. 2A). Due to the precipitation of HIDP–KLH, it was impossible to estimate the degree of conjugation. By the same procedure, we also prepared the HIDP–BSA conjugate as a coating agent for the ELISAs. Since this conjugate was not precipitated, the degree of conjugation of **2** to BSA–SH was estimated by HPLC (Fig. 2B). Peaks *a* and *b* corresponded to HIDP–BSA and unreacted **2**, respectively. On the basis of their peak intensities, approximately 0.59 mg of **2** was found to be conjugated to 1.0 mg of BSA–SH. During the preparation of the monoclonal antibodies, hybridomas were selected on the basis of the ability of their antibodies to bind to the HNE-modified BSA. After repeated screening, three clones were obtained. Among them, the antibody (mAb2C12) produced by the clone 2C12 most strongly recognized the HNE-modified BSA, but did not recognize the native BSA.

Lipid peroxidation yields lipid hydroperoxides as the major initial reaction products whose decomposition generates a number of breakdown products such as aldehydes. It is therefore conceivable that the antibody recognizes epitopes originating from the lipid peroxidation products other than HNE. Hence, we examined the immunoreactivity of mAb2C12 to the proteins, including the aldehyde-modified BSA, ONE-modified BSA and HIDP–BSA, by a direct ELISA. As shown in Fig. 2C, none of the aldehydes, except HNE, generated epitopes recognized by mAb2C12. It was found that the immunoreactivity of the ONE-modified BSA was much higher than that of the HNE-modified BSA. This is consistent with the previous observation by Xu and Sayre [9] that ONE forms the HIDP fluorophore more efficiently than HNE. The mAb2C12

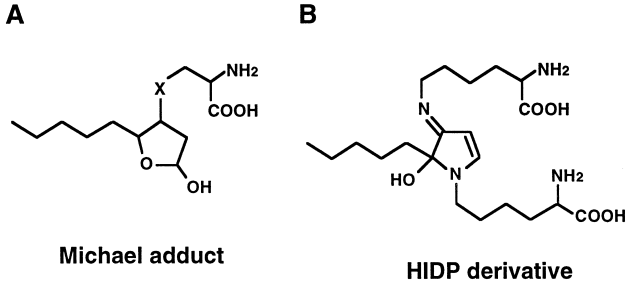

Fig. 1. Chemical structures of Michael-type adducts (A) and the HNE-derived HIDP fluorophore (B). In A, X represents the cysteine, histidine and lysine residues.

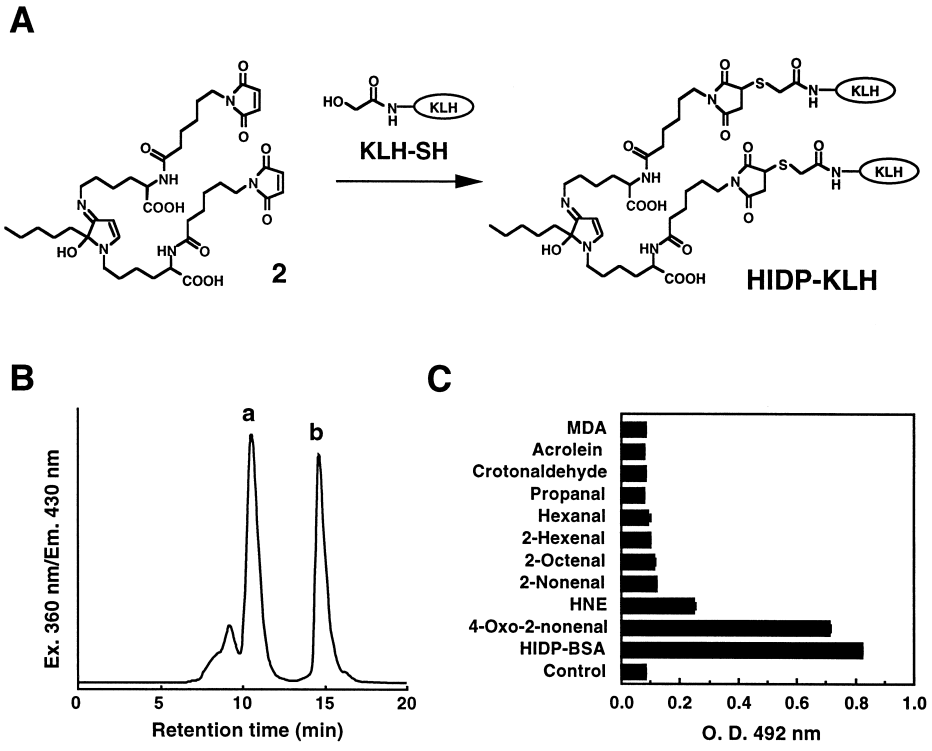

Fig. 2. Preparation of anti-HIDP specific monoclonal antibody (mAb2C12). A: Preparation of the fluorophore-conjugated KLH (HIDP–KLH). B: HPLC profile of the conjugation mixture of maleimide-activated HIDP derivative (**2**) and BSA–SH. HPLC was carried out on a TSK gel SUPER SW 3000 (Tosoh, Japan) eluting with 0.1 M phosphate buffer (pH 7.4) at a flow rate of 0.35 ml/min. Fluorescence detection was with ex/em 360/430 nm. C: Immunoreactivity of aldehyde-modified BSAs and HIDP–BSA conjugate toward mAb12C2. The reaction mixture (0.5 ml) containing aldehyde (1.0 mM) and BSA (1.0 mg/ml) in 0.1 M phosphate buffer (pH 7.4) was allowed to stand at room temperature for 24 h. The mixture was directly subjected to a direct ELISA.

also exhibited a strong recognition of HIDP–BSA. It is noteworthy that HIDP–BSA was not recognized by a monoclonal antibody (mAbHNEJ2) [14] specific to the HNE Michael adducts (data not shown). This ruled out the possibility that mAb2C12 recognizes the HNE Michael adducts.

3.2. *Immunohistochemical detection of the hydoxyiminodihydropyrrole fluorophore in atherosclerotic lesions of human aorta*

Atherosclerotic tissues were immunohistochemically examined for the HIDP derivative using mAb2C12. The abdominal aortas of 65-year-old men were used for the analysis of atherosclerotic lesions. As a control, a 2-year-old girl showing no atherosclerotic lesions either macro- or microscopically was used. Negligible levels of HNE-antibody immunostaining was observed in the aorta of the 2-year-old girl (data not shown). Whereas, the fluorophore was detected in the macrophage-derived foam cells and in the thickening neointima of arterial walls of the 65-year-old man (Fig. 3A). Strong staining in the areas other than the granular cytoplasmic elements of foam cells, may therefore be attributed to HNE modification of the non-cellular components in the surrounding sclerotic stroma. Preabsorption of the antibody with the fluorophore–protein conjugate (50 μg) significantly decreased the immunostaining (Fig. 3B), indicating the specific reactivity of the antibody with the epitope. The detection of the HIDP fluorophore in vivo suggests that the reaction between the lipid peroxidation-derived aldehydes and lysine residues of protein might represent a process common to the formation of the lipofuscin-like fluorophore during aging and its related diseases.

3.3. *Formation of the hydoxyiminodihydropyrrole fluorophore during LDL peroxidation*

Based on the observation that the HIDP derivative has the same fluorescent spectral characteristics (around $\lambda_{ex} = 360$ nm, $\lambda_{em} = 430$ nm) as the oxidized LDL (Fig. 4A), there is a possibility that the HIDP fluorophore contributes to the fluorescence in the oxidized LDL. Incubation of the LDL with Cu^{2+} (24 h, 37°C) led to oxidation of the LDL as assessed by the formation of 2-thiobarbituric acid reactive substances (data not shown). After separation by agarose gel electrophoresis (Fig. 4B, left), the native form of the LDL appears as a single protein band that is readily visualized by Fat Red 7B staining; however, the LDL incubated with 5 μM Cu^{2+} showed an increased electrophoretic mobility, indicating an increased negative charge of the molecule probably due to the modification of the ε-amino group of the lysine residues. An immunoblot analysis of the Cu^{2+}-oxidized LDL using mAb2C12 revealed the formation of immunoreactive materials which were not detected in the native LDL (Fig. 4B, right). Thus, one of the fluorophores generated during the LDL peroxidation was identified to be the HNE-derived HIDP derivative. To further examine the formation of the HIDP fluorophore during the lipid peroxidation reactions, a model experiment of LDL peroxidation was carried out in which linoleic acid was

oxidized with an iron/ascorbate-mediated free radical generating system. As shown in Fig. 4C, the iron/ascorbate-mediated oxidation of linoleic acid in the presence of protein resulted in a time-dependent increase in the antigenicity of protein, whereas the incubation with either iron or ascorbate alone scarcely generated the antigenic materials, suggesting that lipid peroxidation of linoleic acid is essential for production of the fluorophore.

The fact that modification of lysine residues on the surface of the LDL particle is important for the interaction with macrophage-bound receptors suggests that the fluorescent properties of the HIDP fluorophore may also be involved in the internalization of oxidized LDL. In addition, it is notable that the formation of structures analogous to the HIDP derivative in HNE-modified proteins would imply protein cross-linkings. It has been previously reported [15,16] that the HNE cross-linked fluorescent protein is resistant to proteolysis and acts as a potent inhibitor of proteasome, an enzyme complex involved in the degradation of altered proteins. It is thus likely that the formation of the HIDP fluorophore in protein may contribute to the accumulation of the altered forms of protein as a function of age and disease state.

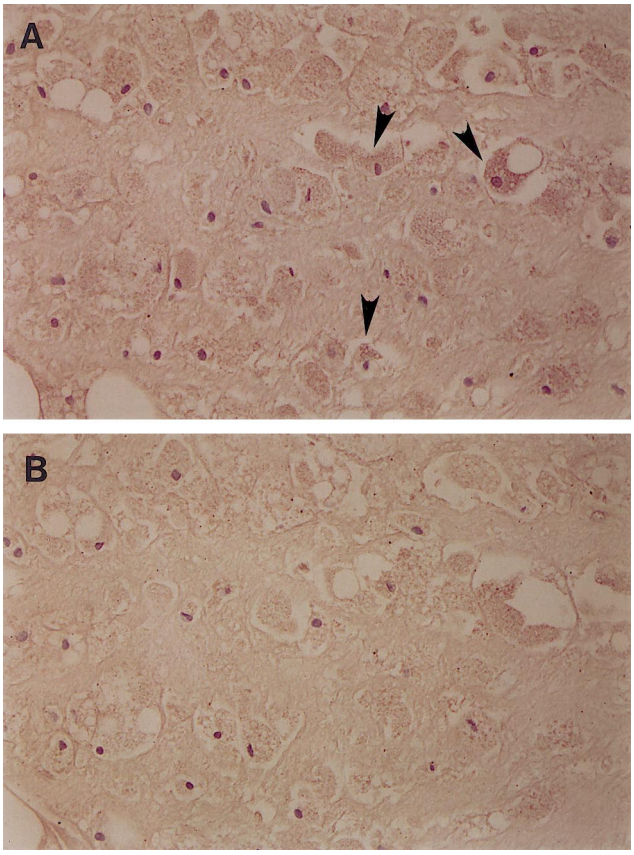

Fig. 3. Immunohistochemical analyses of atheromatous lesions from human abdominal aorta. Immunohistochemical analyses were conducted using the mAb2C12 with the ABC method with alkaline phosphatase. A: Aorta from a 65-year-old man, ×63. A significant immunostaining was observed in the macrophage-derived foam cells (arrowheads). B: For a competitive experiment, mAb2C12 preincubated with an excess of HIDP–BSA was used.

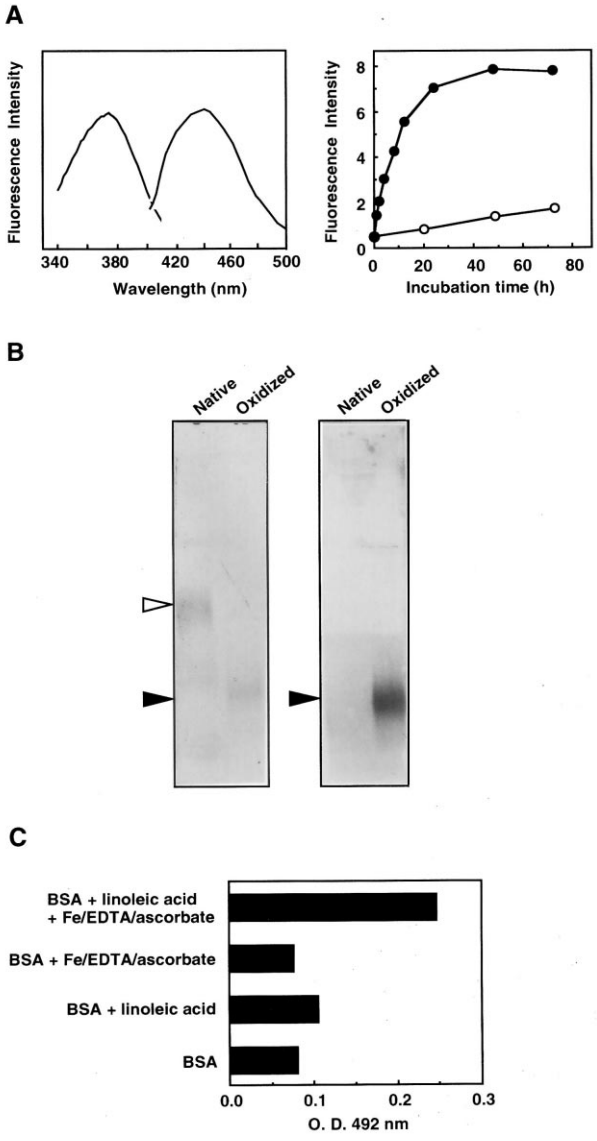

Fig. 4. Formation of the HNE-derived fluorophore during LDL peroxidation. LDL (0.5 mg/ml) was incubated with 5 μM $CuSO_4$ in PBS at 37°C. A: Fluorescence of oxidized LDL: left, fluorescence spectra of Cu^{2+}-oxidized LDL after 24 h; right, time-dependent increase in relative fluorescence intensity of LDL incubated with Cu^{2+}. B: Agarose gel electrophoresis/immunoblot analysis of oxidized LDL: left, agarose gel electrophoresis; right, immunoblot analysis with mAb2C12. Native and oxidized LDL are indicated by open and closed arrowheads, respectively. C: Formation of the HNE-derived fluorophore in BSA treated with linoleic acid in the presence of iron/EDTA/ascorbate.

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