Protein modification by a Maillard reaction intermediate methylglyoxal

Immunochemical detection of fluorescent 5-methylimidazolone derivatives in vivo

Koji Uchida[a,*], Ooi Thiang Khor[a], Tomoko Oya[a], Toshihiko Osawa[a], Yoshinari Yasuda[b], Toshio Miyata[b]

[a]*Laboratory of Food and Biodynamics, Nagoya University School of Agricultural Sciences, Nagoya 464-01, Japan*
[b]*Institute of Medical Sciences and Department of Medicine, Tokai University School of Medicine, Isehara 259-11, Japan*

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Abstract Methylglyoxal (MG), an endogenous metabolite that increases in diabetes, is a common intermediate in nonenzymatic glycation (Maillard reaction) in vivo. Here we describe the immunochemical approach to the detection of MG adducts in proteins in vitro and in atherosclerotic lesions of human aorta in vivo. The reaction of protein (bovine serum albumin) with MG led to selective loss of arginine and lysine residues, accompanied by the formation of 5-methylimidazolone (N^{δ}-(5-methylimidazolon-2-yl)ornithine) and imidazolysine (1,3-di-lysino-4-methylimidazole) derivatives, respectively. The anti-5-methylimidazolone antibody was prepared by immunizing rabbits with a MG-keyhole limpet hemocyanin conjugate and purifying the serum on an affinity gel prepared by covalent attachment of the 5-methylimidazolone derivative. The antibody cross-reacted with the proteins treated with not only MG but trioses, such as hydroxyacetone, dihydroxyacetone, and glyceraldehyde. The immunohistochemical analysis revealed that atherosclerotic lesions of human aorta contained 5-methylimidazolone derivatives whose distributions were identical to those of advanced glycation end products (AGEs) detected by the anti-AGE antibody.

Key words: Methylglyoxal; Maillard reaction; 5-Methylimidazolone; Antibody

1. Introduction

Nonenzymatic glycation (Maillard reaction) is a complex series of reactions between reducing sugars and amino groups of proteins, which leads to browning, fluorescence, and cross-linking of the proteins. The reaction is initiated with the reversible formation of a Schiff's base which undergoes a rearrangement to form a relatively stable Amadori product. The Amadori product further undergoes a series of reactions through dicarbonyl intermediates to form advanced glycation end products (AGEs) [1]. It has been shown that the formation of AGEs in vivo contributes to the pathophysiologies associated with aging and long-term complications of diabetes [2].

A number of sugars, in addition to glucose, are known to form AGEs. Methylglyoxal (MG), among them, has recently received considerable attention as a mediator to form AGEs. MG is a biological metabolite formed by the nonenzymatic and enzymatic degradation of triose phosphate, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. Other sources include aminoacetone, hydroxyacetone, and products of threonine catabolism and acetone metabolism, respectively [3]. It has been reported that the concentration of MG increases in cultured human red blood cells during hyperglycemia [4]. Moreover, increased levels of MG are also found in blood from diabetic patients and in the lens of streptozotosin-induced diabetic rats [5,6]. In vitro experiments have shown that MG undergoes a rapid reaction with proteins [7]. The reactions have been shown to occur even at physiological concentrations of MG [8] and form fluorescent products, characteristics of which resemble those occurring in proteins in aging and diabetes [9]. The high reactivity of MG with proteins and its relatively high concentration in the plasma [10] suggest that MG represents a common intermediate in the formation of AGEs in vivo. This assumption may be supported by the fact that macrophages have a specific receptor for proteins modified by reaction with MG, as well as for glucose-derived AGEs [11].

In the present study, based on the observation that MG adducts of arginine and lysine were predominant products upon in vitro incubation of MG with protein, we raised antibodies directed to the MG adducts and examined their presence in human atherosclerotic lesions in vivo.

2. Materials and methods

2.1. Materials

Keyhole limpet hemocyanin (KLH) was obtained from Pierce. Horseradish peroxidase-linked anti-rabbit IgG immunoglobulin, and ECL (enhanced chemiluminescence) Western blotting detection reagents were obtained from Amersham. Affi-gel 102 was obtained from Bio-Rad. Methylglyoxal and other aldehydic compounds, N^{α}-acetyl-L-lysine, N^{α}-acetyl-L-arginine, and bovine serum albumin (BSA) were obtained from Sigma.

2.2. The MG adducts of N^{α}-acetylarginine and N^{α}-acetyllysine

The reaction mixture (10 ml) contained 100 mM MG and 100 mM amino acid derivative (N^{α}-acetylarginine or N^{α}-acetyllysine) in 50 mM sodium phosphate buffer (pH 7.2). After incubation for 3 days at 37°C, the samples were applied to a Develosil ODS-HG-5 column (8×250 mm) (Nomura Chemicals) equilibrated in a solution of 10% methanol in 50 mM acetic acid and eluted at a flow rate of 1.0 ml/min. The elution profiles were monitored by absorbance at 215 nm. Under these conditions, the major reaction products of MG with *N*-acetylarginine and *N*-acetyllysine were eluted at 9.0 min and 18.0 min, respectively. The chemical structure of the products was confirmed by fast atom bombardment-mass spectrometry (glycerol matrix) on a JEOL JMS-DX 705 mass spectrometer and nuclear magnetic reso-

*Corresponding author. Fax: (81) (52) 789-5296.
E-mail: uchidak@nuagr1.agr.nagoya-u.ac.jp

Abbreviations: MG, methylglyoxal; AGEs, advanced glycation end products; CML, N^{ε}-(carboxymethyl)lysine; KLH, keyhole limpet hemocyanin; ELISA, enzyme-linked immunosorbent assay; HEPES, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; BSA, bovine serum albumin; TBS, Tris-buffered saline

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nance (NMR) on a Bruker ARX-400 spectrometer with tetramethylsilane as the internal standard [8,12]. The two major MG conjugates of arginine and lysine were identified as the 5-methylimidazolone (N^{α}-acetyl-N^{δ}-(5-methylimidazolon-2-yl)ornithine) and the imidazolysine (1,3-di-N^{α}-acetyllysino-4-methylimidazole) derivatives.

2.3. Amino acid analysis

Bovine serum albumin (1 mg/ml) in 50 mM sodium phosphate buffer (pH 7.2) was treated with 100 mM MG at 37°C. After incubation, the reaction mixtures were treated with 10% trichloroacetic acid. After centrifugation at 10000×*g* for 3 min, the proteins were hydrolyzed in vacuo with 6 M HCl for 24 h at 105°C. The hydrolysates were concentrated and dissolved with 50 mM sodium phosphate buffer (pH 7.2). The amino acid analysis was performed with a JEOL JLC-500 amino acid analyzer equipped with a JEOL LC30-DK20 data analyzing system.

2.4. Antibody preparations

Polyclonal antiserum against MG-modified proteins was raised by immunizing a New Zealand White rabbit with KLH that had been treated with MG. In order to prepare a mono-specific antibody that specifically reacts with N^{δ}-(5-methylimidazolon-2-yl)ornithine or 1,3-di-lysino-4-methylimidazole, the antiserum was treated with the affinity gel (Affi-gel 102) which was derivatized by incubation of the gel slurry with N^{α}-acetyl-N^{δ}-(5-methylimidazolon-2-yl)ornithine or 1,3-di-N^{α}-acetyllysino-4-methylimidazole in 0.1 M HEPES, pH 8.0, for 20 h at 4°C. The antiserum (2 ml) was loaded onto the column containing 1 ml of affinity resin. Unbound proteins were removed by washing with 40 ml of 0.1 M HEPES buffer, pH 8.0, followed by 20 ml of 100 mM NaCl, and the bound antibodies were eluted with 20 ml of 0.1 M glycine, pH 2.5. The eluate with glycine was immediately neutralized with 1 M Tris-HCl, pH 8.0, and stored at −80°C.

2.5. Enzyme-linked immunosorbent assay (ELISA)

A coating antigen was prepared by incubating 1 mg of BSA with 10 mM aldehyde in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, for 24 h at 37°C. A 100-μl aliquot of the antigen solution containing 0.4-mg protein 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 filled with 200 μl of Block Ace solution (100 mg/ml) for 30 min at 37°C. The primary antibody was then added to the wells at 100 μl/well of 1 μg/ml solution for 3 h at 37°C or overnight at 4°C. The plate was then washed once with TBS/Tween. After discarding the supernatants and washing three times with TBS/Tween, 100 μl of a 5×10^{3} dilution of goat anti-rabbit IgG 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. Enzyme-linked antibody bound to the well was revealed by adding 100 μl/well of 1,2-phenylenediamine in 0.1 M citrate/phosphate buffer (pH 5.0) containing 0.003% H_2O_2. The reaction was terminated by the addition of 50 μl of 2 M sulfuric acid, and the absorbance at 492 nm was read on a micro-ELISA plate reader.

2.6. Imunoblot analysis

The samples were treated with Laemmli sample buffer [13] for 3–5 min at 100°C. The samples containing 10-μg protein were run on two 12% SDS-PAGE slab gels. One gel was used for staining with Coomassie brilliant blue; the other was transblotted to nitrocellulose membranes, incubated with 2% BSA in TBS/Tween for blocking, washed, and treated with the primary antibody. This procedure was followed by the addition of horseradish peroxidase conjugated to goat anti-rabbit IgG immunoglobulin and ECL reagents. The bands were visualized by the exposure of the membranes to autoradiography film.

2.7. Immunohistochemistry

For indirect immunohistochemical staining, human arterial tissues obtained at autopsy were cut into 5-μm sections, mounted on the slides coated with 3-aminopropyltriethoxy silane, deparaffined, rehydrated in distilled water, and then blocked in 4% skim milk for 2 h. The sections were incubated with either anti-5-methylimidazolone rabbit IgG (5 μg/ml), anti-imidazolysine rabbit IgG (5 μg/ml), anti-AGE mouse monoclonal IgG (2 μg/ml) (6D12) (Wako Pure Chemicals) [14], or anti-CD68 mouse monoclonal IgG (2 μg/ml) (Dako) overnight in humid chambers at room temperature. The sections were washed and incubated with 1/100 diluted goat anti-rabbit or anti-mouse IgG conjugated with peroxidase (Dako) for 2 h at room temperature, followed by detection with 3,3′-diaminobenzidine solution containing 0.003% H_2O_2. Competition experiments to confirm the specificity of immunostaining were also performed with anti-5-methylimidazolone IgG which was preincubated for 4 h at 37°C with an excess of free N^{α}-acetyl-N^{δ}-(5-methylimidazolon-2-yl)ornithine. Non-immune rabbit or mouse IgG was used as a negative control.

3. Results

3.1. Characterization of MG conjugates of arginine and lysine

As consistent with the previous findings [15–17], the reaction of protein (BSA) with MG resulted in selective loss of arginine and lysine residues. Accompanied with the irreversible modification of these amino acids, we detected two products (compounds **1** and **2**), which were probably ascribed to the formation of MG-arginine and/or MG-lysine adducts, in the hydrolysates of MG-modified proteins (Fig. 1). To determine the structure of MG-modified arginine and lysine residues in proteins, the products formed by the reaction of MG with N^{α}-acetylarginine or N^{α}-acetyllysine were examined. Reaction of *N*-acetylarginine with MG generated a main product which was identified as the 5-methylimidazolone derivative, N^{α}-acetyl-N^{δ}-(5-methylimidazolon-2-yl)ornithine (Fig. 2A), whose amino acid analysis gave a single peak identical to compound **1**. Whereas the reaction of N^{α}-acetyllysine with MG selectively generated the imidazolysine derivative, 1,3-di-N^{α}-acetyllysino-4-methylimidazole (Fig. 2B), which gave compound **2** after acid-hydrolysis. As shown in Fig. 3, when BSA (1 mg/ml) was treated with 100 mM MG at 37°C, the level of both MG adducts reached a maximum within 24 h.

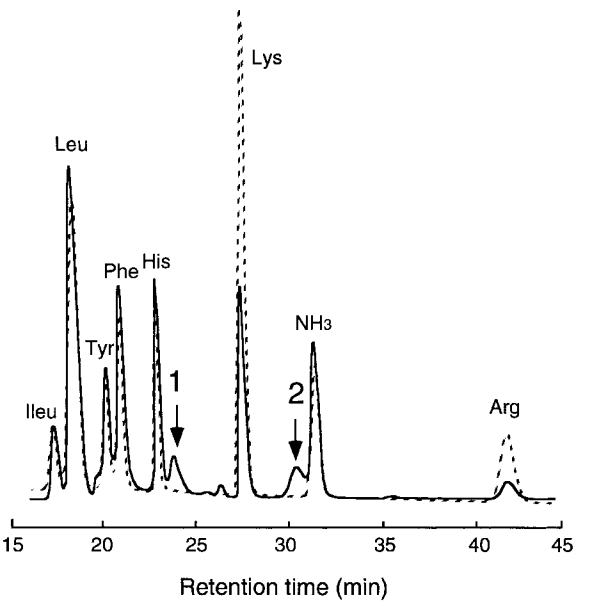

Fig. 1. Detection of MG adducts by amino acid analysis. BSA (1 mg/ml) in 50 mM sodium phosphate buffer (pH 7.2) was incubated in the absence (dotted line) and presence (solid line) of 100 mM MG at 37°C. After incubation, the reaction mixtures were treated with 10% trichloroacetic acid. After centrifugation at 10000×*g* for 3 min, the proteins were hydrolyzed and subjected for automated amino acid analysis. The products **1** and **2** represent the newly formed MG adducts.

3.2. Antibodies raised against MG-treated KLH

To detect the 5-methylimidazolone and imidazolysine derivatives in biological tissue samples, antibodies were raised by immunizing rabbits with KLH directly modified with MG. Affinity purification using the 5-methylimidazolone or imidazolysine derivative as the ligand revealed that the anti-MG/KLH antiserum contained antibody populations specific to these derivatives. The immunoblot analysis using the anti-5-methylimidazolone IgG revealed that the immunoreactive materials were produced upon the reaction of BSA with MG (Fig. 4A), while the anti-imidazolysine IgG showed a weak immunoreactivity with the MG-modified BSA (data not shown). It was also revealed that the anti-5-methylimidazolone IgG recognized not only the MG-modified proteins but the proteins treated with the trioses, including hydroxyacetone, dihydroxyacetone, and glyceraldehyde (Fig. 4B).

3.3. Presence of 5-methylimidazolone derivative in the atherosclerotic lesions of human aorta

It has been demonstrated that the rate of MG production increases during hyperglycemia [18]. Hence, in the present study, the presence of 5-methylimidazolone derivatives in vivo was evaluated in human atherosclerotic lesions by immunohistochemistry. Massive deposits observed in the tissue were stained for the affinity-purified IgG to the 5-methylimidazolone derivative (Fig. 5A). The deposits were surrounded with a number of mononuclear cells that were stained positive for CD68 (Fig. 5B), indicating that the infiltrating cells were tissue macrophages. It is of interest to note that the staining patterns of 5-methylimidazolone in these deposits were almost identical with those of the AGEs (Fig. 5C). Preadsorption of affinity-purified 5-methylimidazolone IgG by free N^{α}-acetyl-N^{δ}-(5-methylimidazolon-2-yl)ornithine abolished the immunostaining (Fig. 5D), indicating the specific reactivity of the antibody with epitopes. Non-immune rabbit or mouse IgG gave no immunostaining pattern. These results suggest that MG forms adducts with arginine and accumulates in the atherosclerotic lesions, although the physiological significance of the formation of MG adducts, most importantly whether or not they have specific atherogenic properties, remains to be established.

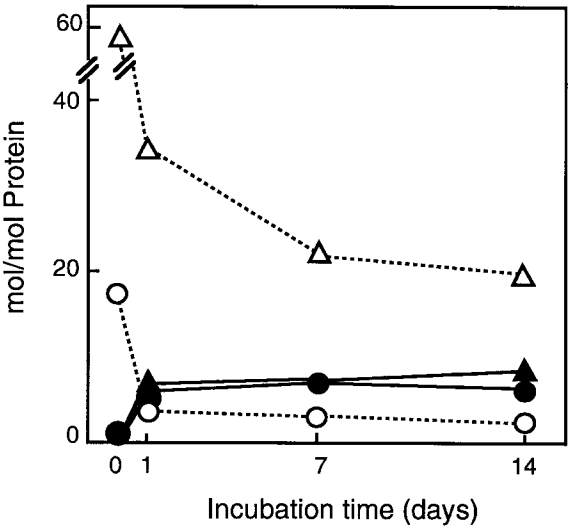

Fig. 3. Stoichiometry of loss of amino acids (arginine and lysine) and formation of MG adducts. Symbols: ○, arginine; △, lysine; ●, MG-arginine adduct (N^{δ}-(5-methylimidazolon-2-yl)ornithine); ▲, MG-lysine adduct (1,3-dilysino-4-methylimidazole).

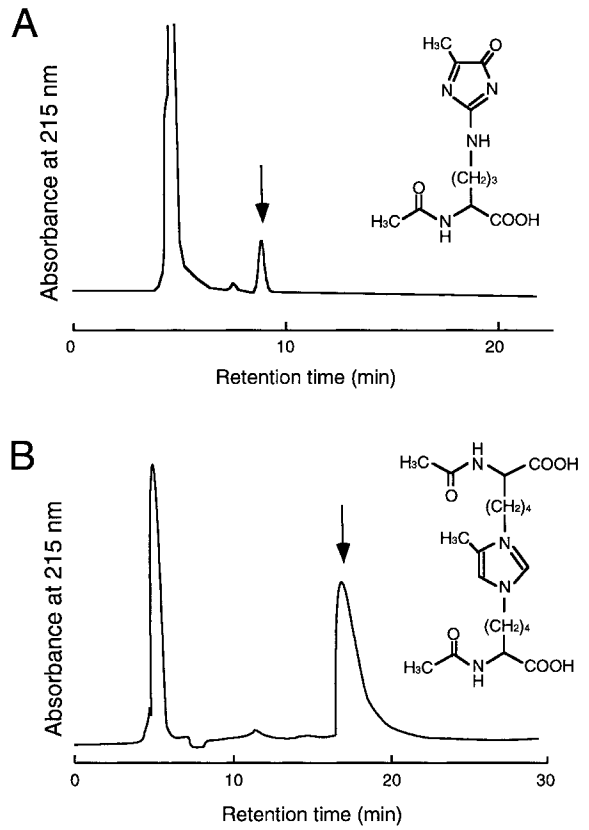

Fig. 2. HPLC profiles of the reaction mixtures of MG/*N*-acetylarginine (A) and MG/*N*-acetyllysine (B). The reaction mixture (10 ml) contained 100 mM MG and 100 mM amino acid derivative (N^{α}-acetylarginine or N^{α}-acetyllysine) in 50 mM sodium phosphate buffer (pH 7.2). After incubation for 3 days at 37°C, the samples were applied to a Develosil ODS-HG-5 column (8×250 mm) equilibrated in a solution of 10% methanol in 50 mM acetic acid and eluted at a flow rate of 1.0 ml/min. Arrows indicate major products identified as N^{α}-acetyl-N^{δ}-(5-methylimidazolon-2-yl)ornithine (A) and 1,3-di-N^{α}-acetyllysino-4-methylimidazole (B).

4. Discussion

As previously reported [15–17], arginine and lysine residues represented major targets of MG in proteins, which resulted in irreversible modification of these amino acids. We demonstrated that the irreversible modification of arginine and lysine by MG was partially ascribed to the formation of 5-methylimidazolone and imidazolysine derivatives, respectively (Figs. 1–3). To detect these MG adducts in biological tissue samples, immunochemical procedures using a specific antibody to the adducts were made. The affinity purification using the 5-methylimidazolone derivative as the ligand enabled to separate antibody populations specific to these derivatives from the antiserum. Using this antibody, we demonstrated for the first time the formation of 5-methylimidazolone derivatives in vivo. The data suggested that the formation of MG followed by the irreversible modification of proteins was associated with the development of atherosclerosis. Although the formation of 5-methylimidazolone in other tissues remains to be

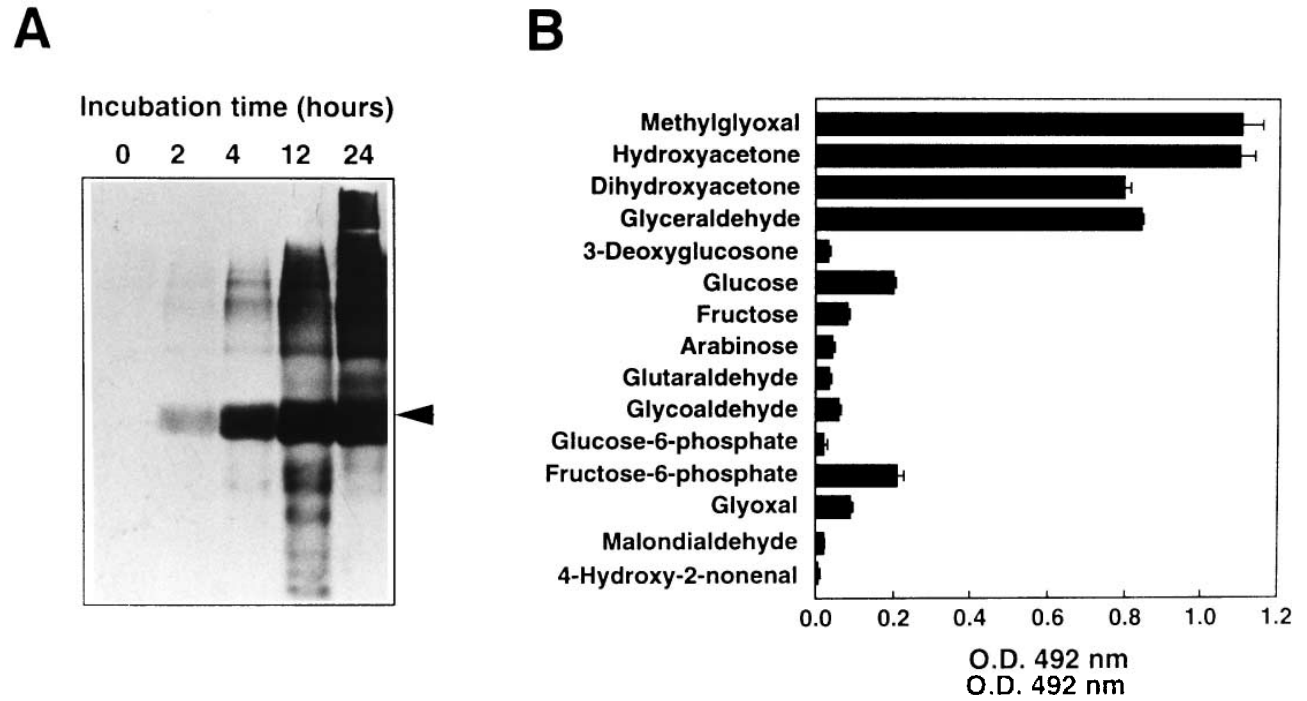

Fig. 4. Immunoreactivity of anti-5-methylimidazolone IgG with modified proteins. (A) Immunoblot analysis of BSA treated with MG. BSA (1 mg/ml) in 50 mM sodium phosphate buffer (pH 7.2) was incubated with 10 mM MG at 37°C. Formation of MG adducts in the protein was determined by immunoblot analysis using the affinity-purified anti-5-methylimidazolone IgG. (B) ELISA analysis of BSA treated with aldehydic compounds. A coating antigen for ELISA was prepared by incubating 1 mg of BSA with 10 mM aldehydic compound in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, for 24 h at 37°C.

established, this is expected to be shown in kidney, lens, and plasma proteins in diabetes mellitus, commensurate with the increase in MG in the tissue and blood concentration [4–6].

Lo et al. [8] have reported that the reaction of MG with arginine residues proceeds via the reversible formation of a glycosylamine and a dihydroxyimidazolidine, with slow conversion to form a fluorescent imidazolone derivative. The formation of imidazolone has been suggested to involve the spontaneous autoxidation of the intermediate 1,5-dihydroxyimidazolone. This and the fact [19] that the reaction of MG with amino acids generates free radicals imply that MG production may be closely associated with oxidative stress. We have observed that, in human atherosclerotic lesions, the immunoreactive materials with the affinity-purified anti-5-methylimidazolone IgG colocalized with those detected with the antibody to the modified proteins with lipid peroxidation products, such as 4-hydroxy-2-nonenal and malondialdehyde (T. Miyata, Y. Yasuda and K. Uchida, unpublished observation). The importance of these lipid peroxidation-derived aldehydes has been implicated in the pathogenesis of numerous diseases, including atherosclerosis [20], cancer [21], Parkinson's disease [22], and Alzheimer disease [23,24], leading to the assumption that MG represents one of the primary sources of oxidative damage to protein in complex biological matrices.

It has been suggested that the imidazolones represent common structures in advanced glycation reactions [25]. In vitro glycation of proteins in fact generates an imidazolone derivative, N^{δ}-(5-(2,3,4-trihydroxybutyl)-4-imidazolon-2-yl)ornithine [26]. The previous finding [11] that a macrophage receptor for MG-modified proteins recognizes glucose-modified proteins also suggests the possibility that the imidazolones are formed commonly as AGEs. The observation (Fig. 4B) that the anti-5-methylimidazolone IgG cross-reacted with proteins that had been treated with trioses, such as hydroxyacetone, dihydroxyacetone, and glyceraldehyde, suggested that these compounds produced similar immunoreactive 5-methylimidazolone derivatives in the protein to those generated in the MG-modified protein. In fact, proteins modified with dihydroxyacetone produce a derivative with identical fluorescence characteristics to MG-modified proteins [9]. Alternatively, they may produce MG during incubations, leading to the formation of MG-modified proteins. This may be supported by the previous observations that triose phosphates such as glyceraldehyde-3-phosphate and dihydroxyacetonephosphate degrade to MG [27,28]. Other dicarbonyl intermediates such as 3-deoxyglucosone could also form imidazolones [29]; however, the anti-5-methylimidazolone IgG scarcely cross-reacted with the protein treated with 3-deoxyglucosone (Fig. 4B).

The immunohistochemical analysis using the antibody to 5-methylimidazolone revealed that atherosclerotic lesions contained epitopes in the granular cytoplasmic elements of foam cells (Fig. 5). Westwood et al. [11] have reported that both MG-modified proteins and glucose-derived AGEs bind to a common receptor in macrophages. The intracellular granular staining observed in atherosclerotic lesions may, therefore, represent the presence of MG-derived AGEs that had already been taken up by macrophages and are present within the cell in cytoplasmic organelles. Recently, one of the major epitopes of the anti-AGE antibody has been identified as N^{ε}-(carboxymethyl)lysine (CML) which is formed upon the reaction of lysine residues with glyoxal generated during the glycation reaction. In addition, CML has also been reported as a product of lipid peroxidation [30]: a mechanism has been proposed in which the metal-catalyzed oxidation of polyunsaturated fatty acids in the presence of protein leads to the formation

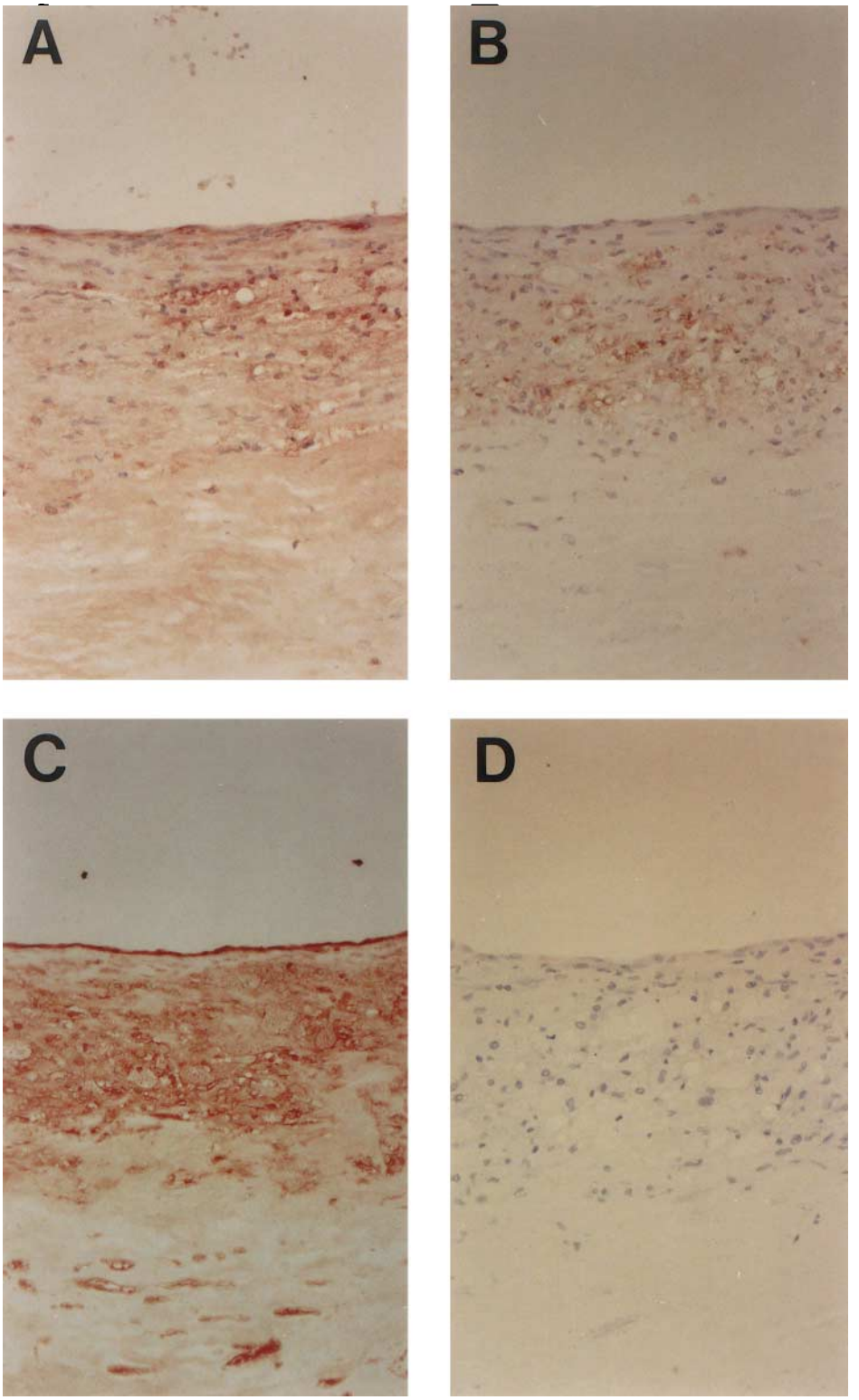

Fig. 5. Immunohistochemical detection of 5-methylimidazolone derivatives and AGEs in fatty streak lesions of arterial tissue. Arterial tissue specimen from a 69-year-old male with atherosclerosis was immunostained for 5-methylimidazolone (A and D), CD68 (B), or AGEs (C). For a competitive experiment, anti-5-methylimidazolone IgG preincubated with an excess of N^{α}-acetyl-N^{δ}-(5-methylimidazolon-2-yl)ornithine was used (D). The nuclei were counterstained with Meyer's hematoxylin. The staining for 5-methylimidazolone and AGEs was positive in fatty streak lesions. A–D, ×200.

of glyoxal, an intermediate formed during lipid peroxidations, reacting with lysine residues to generate CML. The observation that the staining patterns of 5-methylimidazolone in these deposits were almost identical with those of the AGEs (Fig. 5C) suggested that dicarbonyl intermediates, including MG and glyoxal, play an important role in the formation of arte-

rial foam cells and contribute to the development of atherosclerosis.

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