J. Biochem. **136**, 351–358 (2004)
DOI: 10.1093/jb/mvh124

N^{ε}-(Carboxymethyl)lysine and 3-DG-Imidazolone Are Major AGE Structures in Protein Modification by 3-Deoxyglucosone

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Received April 21, 2004; accepted June 14, 2004

The levels of plasma 3-deoxyglucosone (3-DG) increase under hyperglycemic conditions and are associated with the pathogenesis of diabetic complications because of the high reactivity of 3-DG with proteins to form advanced glycation end products (AGE). To investigate potential markers for 3-DG–mediated protein modification *in vitro* and *in vivo*, we compared the yield of several 3-DG–derived AGE structures by immunochemical analysis and HPLC and measured their localization in human atherosclerotic lesions. When BSA was incubated with 3-DG at 37°C for up to 4 wk, the amounts of N^{ε}-(carboxymethyl)lysine (CML) and 3-DG-imidazolone steeply increased with incubation time, whereas the levels of pyrraline and pentosidine increased slightly by day 28. In contrast, significants amount of pyrraline and pentosidine were also observed when BSA was incubated with 3-DG at 60°C to enhance AGE-formation. In atherosclerotic lesions, CML and 3-DG-imidazolone were found intracellularly in the cytoplasm of most foam cells and extracellularly in the atheromatous core. A weak-positive immunoreaction with pyrraline was found in the extracellular matrix and a few foam cells in aortic intima with atherosclerotic lesions. Our results provide the first evidence that CML and 3-DG-imidazolone are major AGE structures in 3-DG–modified proteins, and that 3-DG-imidazolone provides a better marker for protein modification by 3-DG than pyrraline.

Key words: 3-deoxyglucosone (3-DG), 3-DG-imidazolone, N^{ε}-(carboxymethyl)lysine (CML), pyrraline, advanced glycation end products (AGEs).

Abbreviations: 3-DG, 3-deoxyglucosone; 3DG-BSA, 3-deoxyglucosone–modified BSA; AGE(s), advanced glycation end products; AGE-BSA, glucose-modified AGE–bovine serum albumin; BSA, bovine serum albumin; CML, N^{ε}-(carboxymethyl)lysine; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; ELISA, enzyme-linked immunosorbent assay; MG-BSA, methylglyoxal-modified BSA; PBS, phosphate-buffered saline; RP-HPLC, reverse phase high performance liquid chromatography.

Reducing sugars such as glucose and ribose react nonenzymatically with protein amino residues to form advanced glycation end products (AGEs) of the Maillard reaction through the formation of a Schiff base and Amadori products. AGEs show some physicochemical properties such as fluorescence, browning, and intra- or intermolecular cross-linking. The accumulation of AGEs in long-lived proteins such as lens crystallins (*1*) and collagen (*2*) has been hypothesized to contribute to the development of pathologies associated with aging, diabetes mellitus (*3*), dialysis-related amyloidosis (*4*), and Alzheimer's disease (*5*).

Several aldehydes, such as 3-deoxyglucosone (3-DG) (*6*), glycolaldehyde (*7*, *8*), glyoxal (*9*) and methylglyoxal (*6*), are generated in the Maillard reaction and contribute further to the formation of AGEs. Thornalley *et al*. (*6*) demonstrated that 5 μM of 3-DG was generated by the degradation of 50 mM glucose over 3 wk. Furthermore, 18 μM of 3-DG was detected when 50 mM glucose was incubated for 5 d with 50 mM N^{α}-*t*-BOC-L-lysine, and the level was enhanced with increasing phosphate concentrations. 3-DG reacts rapidly with protein amino residues to form various AGE structures such as N^{ε}-carboxymethyllysine (CML) (*10*, *11*), 3-DG-imidazolone (*12*), pyrraline (*13*), and very minor products such as pentosidine (*14*). Niwa *et al*. (*15*) demonstrated that 3-DG-imidazolone accumulates in nodular lesions and expands the mesangial matrix of glomeruli and renal arteries in advanced stages of diabetic nephropathy as well as in atherosclerotic lesions of the aorta. Pyrraline have been detected in sclerosed glomeruli of patients with diabetes, elderly non-diabetic individuals, arteries with arteriolosclerosis and perivascular and peritubular sclerosed extracellular matrix and basement membrane (*16*). These reports suggest that 3-DG plays an important role in the pathogenesis of diabetic complications. In these 3-DG–derived AGE structures, both 3-DG-imidazolone and pyrraline are reported to be generated specifically from protein modifications by 3-DG, and may serve as potential markers for the presence of 3-DG–modified proteins *in vivo*.

Nagaraj *et al*. (*17*) detected 1,354 pmol of pyrraline/mg protein when bovine α-crystallin (100 mg/ml) was incubated *in vitro* at 37°C for 19 d with 100 mM 3-DG. They

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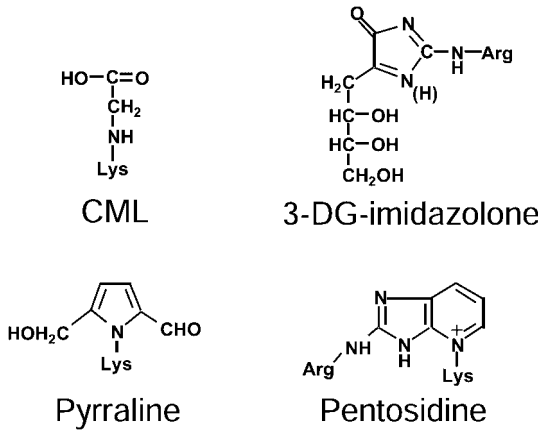

Fig. 1. **Structures of AGEs.**

concluded that pyrraline is a major 3-DG–derived AGE-structure without comparing the amount of pyrraline with the other AGE structures such as CML and 3-DG-imidazolone. On the other hand, Smith *et al.* (*18*) argued that pyrraline is neither a major AGE product *in vivo* nor an early-stage product of the Maillard reaction *in vitro*. Taken together, it is controversial which is the main AGE structure formed by protein modification with 3-DG. In order to clarify this issue, we compared the yield of several AGE structures such as CML, pyrraline, 3-DG-imidazolone and pentosidine (Fig. 1) in 3-DG-modified BSA by enzyme-linked immunosorbent assay (ELISA) as well as HPLC analysis. In addition, to investigate the role of 3-DG–derived AGE structures in atherosclerosis lesions, we examined their immunohistochemical localization in atherosclerosis lesions. Our results clearly demonstrate that CML and 3-DG-imidazolone are generated in the early stage of protein modification by 3-DG. Since CML is generated not only by 3-DG modification but also by modification by glyoxal and the cleavage of the Amadori product, 3-DG-imidazolone can be an important marker for 3-DG–modified proteins *in vivo*.

MATERIALS AND METHODS

Materials—D-Glucose, fatty acid–free bovine serum albumin (BSA) and human serum albumin (HSA) were purchased from Sigma (St. Louis, MO). L-Amino acids (Sigma Ultra, > 99%), N^{α}-*t*-BOC-ornithine, N^{α}-*t*-BOC-arginine, N^{α}-*t*-BOC-lysine, pronase E, prolidase and leucine aminopeptidase (type VI from porcine kidney) were also purchased from Sigma (Poole, UK). Keyhole limpet hemocyanin (KLH) was purchased from Wako (Osaka, Japan). Horseradish peroxidase (HRP)–conjugated goat anti–mouse IgG antibody was purchased from Kirkegaard Perry Laboratories (Gaitherburg, MD, USA). All other chemicals were of the best grade available from commercial sources.

Modification of BSA with 3-DG—3-DG was prepared as described previously (*19*) and the structure was confirmed by proton NMR. 3-DG–modified BSA (3-DG-BSA) was prepared by incubating 2 mg/ml of BSA (1.5 mM lysine residues) with 200 mM of 3-DG at 37°C for up to 4 wk. Since AGE formation is enhanced in a temperature dependent manner during the incubation of a protein with reducing sugars (*20–22*), BSA was also incubated with 200 mM 3-DG at 60°C for up to 1 wk. Aliquots of samples were taken from each reaction mixture and dialyzed against phosphate-buffered saline (PBS).

Chromatographic Assay of AGE—CML, 3-DG-imidazolone, pyrraline and pentosidine in the sample were measured by HPLC as described previously (*23*). The following standard AGE were prepared according to the methods described previously: CML (*24*), 3-DG-imidazolone (*23*), pyrraline (*25*), pentosidine (*26*). For enzymatic hydrolysis, all procedures were carried out under nitrogen. An aliquot of protein solution (100 μg protein) was diluted with 20 μl of water. Aliquots of 40 mM HCl (25 μl), pepsin solution (2 mg/ml in 20 mM HCl; 5 μl), and thymol solution (2 mg/ml in 20 mM HCl; 5 μl), were added and the sample incubated at 37°C for 24 h. The sample was then neutralized and buffered at pH 7.4 by the addition of 25 μl of 0.5 M potassium phosphate buffer (pH 7.4) and 5 μl of 260 mM KOH. Pronase E (2 mg/ml in 10 mM KH_2PO_4, pH 7.4; 5 μl) was added and the sample was incubated at 37°C for 24 h. Aminopeptidase solution (2 mg/ml in 10 mM KH_2PO_4, pH 7.4; 5 μl) and prolidase solution (2 mg/ml in 10 mM KH_2PO_4, pH 7.4; 5 μl) were added to the sample and the sample was incubated at 37°C for 48 h. Aliquots of enzymatic hydrolysate (50 μl, equivalent to 50 μg protein) were placed in 1 ml glass vials and an internal standard (α-aminobutyric acid, 100 nmol/ml; 10 μl) was added, together with water (40 μl), aminoquinolyl-*N*-hydroxysuccimidyl-carbamate (AQC) derivatizing buffer (500 mM borate buffer and 400 μM DTPA, pH 8.8; 100 μl) and AQC (10 mM in acetonitrile; 200 μl). Calibration standards contained 0–1,000 pmol of the AGE standards and 0–20 nmol of the amino acids. Test and calibration standard samples were incubated at 55°C for 10 min. The samples were then lyophilized to dryness *in vacuo* and reconstituted in water (100 μl). AQC-labeled hydrolysates were filtered by centrifugal filtration (0.2 μm pore) and the filtrates were analyzed by analytical reversed phase HPLC. HPLC analysis was performed with a Waters 717 plus autosampler (with samples maintained at 18°C), Waters 600 quaternary pump, Waters 474 fluorescence detector and Waters 481 Lambda Max absorbance detector in series, with a 2-channel data collection system (Kontron) at a flow rate 1 ml/min. Calibration curves of amino acids (0–20 nmol) and AGE, (0–1 nmol) were constructed, and 25 μg of control and glycated proteins were analyzed. The detection limits were 2–20 pmol and the interbatch coefficients of variation were 4–29% depending on the analyte, and the analytical recoveries exceeded 90%.

Preparation of Monoclonal Antibodies against Pyrraline and 3-DG-Imidazolone—Monoclonal antibodies against CML (6D12) (*27*), 3-DG-imidazolone (JNH27) (*28*), and pentosidine (1C12) (*29*) were prepared as described previously. In this study, we prepared the monoclonal anti-pyrraline antibody (*16*) with minor modifications. Caproyl pyrraline was prepared as described previously (*13*). To prepare immunogen, 6 mg of caproyl pyrraline was incubated with 6 mg of KLH in the presence of 50 mM of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Pierce) and 2.5 mM of *N*-hydroxysulfosuccinim-

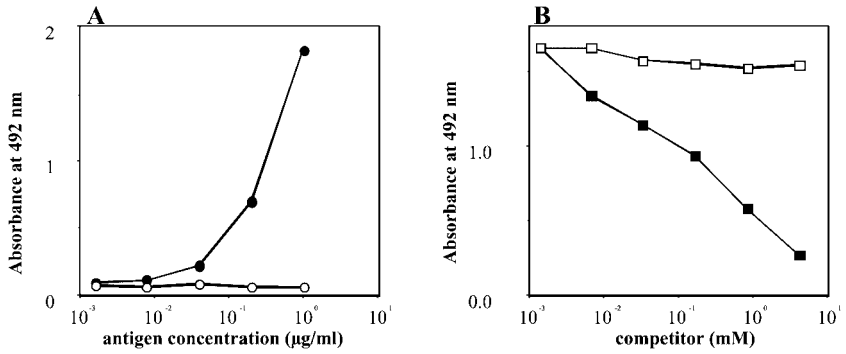

Fig. 2. **Immunoreactivity of the monoclonal anti-pyrraline antibody (H-12).** The immunoreactivity of H-12 was determined by non-competitive ELISA (A) and competitive ELISA (B). For non-competitive ELISA, wells were coated with 100 μl of the indicated concentration of pyrraline-HSA (closed circles) or HSA (open circles). The wells were washed and blocked with gelatin, followed by a 1 h-reaction with 100 μl of 1 μg/ml of H-12. The antibodies bound to wells were visualized by HRP-conjugated anti-mouse IgG as described under "MATERIALS AND METHODS." For competitive ELISA, the wells were coated with pyrraline-HSA (1 μg/ml) and incubated for 1 h with a mixture of 50 μl of caproyl pyrraline (closed circles) or caproic acid (open squares) and 50 μl of H-12 (2 μg/ml), followed by reaction with HRP-conjugated anti-mouse IgG.

ide (Pierce) at room temperature for 1 h. The reaction was terminated by the addition of 20 mM of 2-mercaptoethanol and the sample was dialyzed against PBS for 24 h at 4°C. A monoclonal antibody against pyrraline was prepared as described previously (*16*) with minor modifications. Briefly, splenic lymphocytes from a Balb/c mouse immunized with caproyl pyrraline–conjugated HSA (pyrraline-HSA) was fused with myeloma P3U1 cells. Hybridoma cells positive to caproyl pyrraline–conjugated KLH (pyrraline-KLH) but negative to HSA and KLH were selected through successive subcloning. One cell line, termed H-12, was produced from the ascitic fluid of Balb/c mice and further purified on protein G–immobilized Sepharose gel to IgG_1.

Enzyme-Linked Immunosorbent Assay (ELISA)—For non-competitive ELISA, the wells of a 96-well microtiter plate (Nippon Inter Med, Tokyo) were each coated with 0.1 ml of 0.1–10 μg/ml of samples in 50 mM sodium carbonate buffer (pH 9.6), and incubated for 1 h. The wells were washed three times with PBS containing 0.05% Tween 20 (buffer A), and then blocked with 0.5% gelatin in 50 mM sodium carbonate buffer (pH 9.6) for 1 h. After washing three times with buffer A, the wells were incubated with the indicated concentration of primary antibody for 1 h, followed by incubation with HRP-conjugated anti–mouse IgG antibody, and by reaction with 1,2-phenylendiamine dihydrochloride. The reaction was terminated by the addition of 100 μl of 1.0 M sulfuric acid, and the absorbance at 492 nm was read on micro-ELISA plate reader. For competitive ELISA, each well of an ELISA plate was coated for 1 h with 100 μl of 1.0 μg/ml of pyrraline-HSA or 3-DG–imidazolone-HSA. The wells were washed three times with buffer A, blocked for 1 h with 0.5% gelatin in 50 mM sodium carbonate buffer (pH 9.6), and washed three times with buffer A. Then, 50 μl of the sample to be tested and the same volume of primary antibody were added to each well, and the plate was incubated for 1 h. The antibodies bound to wells were detected by HRP-conjugated anti-mouse IgG antibody.

Tissue Preparation—Sections of arterial walls were obtained from ten males and four females at autopsy within 6 h of death (mean age, 63 + 11.1 yr: range, 43–78 yr). All subjects were both clinically and pathologically free of diabetes mellitus and chronic renal failure. Samples for immunohistochemical analysis comprised 12 grossly normal regions (diffuse initimal thicking, DIT), 8 fatty streaks (FS) and 10 atherosclerotic plaques (AP). For immunohistochemistry, tissue samples were fixed in 2% periodate-lysine-paraformaldehyde at 4°C for 6 h, and washed with phosphate-buffered saline (pH 7.2) containing a graded series of sucrose (10%, 15%, and 20%). After immersion in PBS containing 10% glycerol and 20% sucrose to inhibit the formation of ice crystals, the tissues were embedded in OCT compound (Sakura Fine Technical Co., Ltd., Tokyo, Japan), frozen in liquid nitrogen, and stored at –80°C until use. Sections were cut 5-μm thick with a cryostat MICROM (HM-500M; Walldorf, Germany), and mounted onto poly-L-lysine–coated slides.

Immunohistochemistry—For immunohistochemical analysis of AGE accumulation in human atherosclerotic lesions, cryostat sections were prepared and examined by the indirect immunoperoxidase method using monoclonal anti-AGE antibodies specific for CML, 3-DG-imidazolone, and pyrraline. Briefly, after the inhibition of endogenous peroxidase activity (*30*), the sections were incubated with monoclonal anti-AGE antibody (6D12, 1 μg/ml; JNH-27, 3 μg/ml; H-12, 5 μg/ml; and 1C12, 1 μg/ml), washed with PBS, and reacted with peroxidase-labeled anti-mouse $F(ab')_2$ (Amersham, Poole, UK) diluted 1:100 as the second antibody. After visualization with 3,3′-diaminobenzidine (Dojin Chemical Co., Kumamoto, Japan), the sections were counterstained with hematoxylin and mounted in Malinol (Mutoh Chemical Co., Tokyo, Japan). For negative controls, the same procedures were performed, but the first antibody was omitted. Nonimmune mouse IgG_1 was also used as a negative control, and the staining results showed no immunoreaction. Counterstaining was performed using Mayer's hematoxylin.

RESULTS

Immunoreactivity of the Monoclonal Anti-Pyrraline Antibody—After successive screenings, one cell line, named H-12, that was positive to pyrraline-KLH but negative to HSA and KLH was obtained. As shown in Fig. 2A, H-12 significantly reacted with pyrraline-HSA in a dose-dependent manner, whereas its reactivity with HSA was negligible. Furthermore, competitive ELISA showed that the immunoreaction of H-12 to pyrraline-HSA was significantly inhibited by caproyl pyrraline but not by caproic acid (Fig. 2B). It is likely therefore that the anti-pyrraline antibody recognizes the pyrrole structure.

Time Course of 3-DG–Derived AGE Formation—To determine the effect of incubation time on AGE forma-

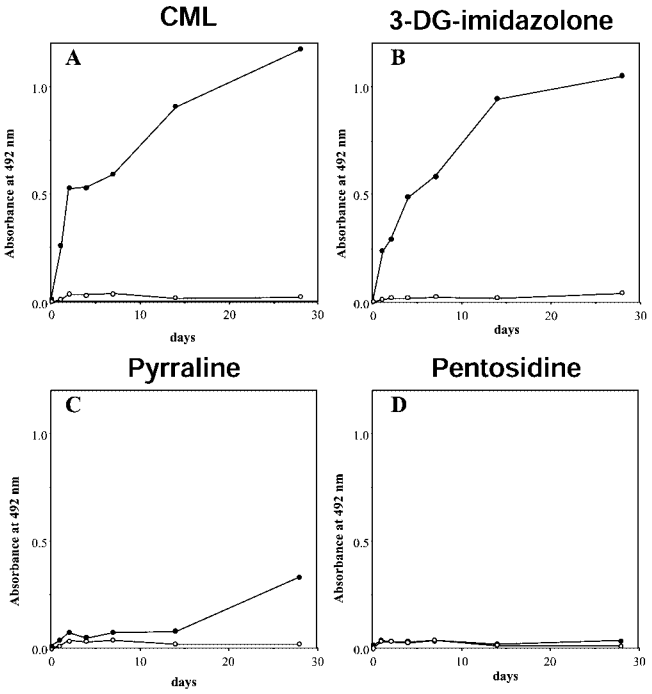

Fig. 3. **Time course of 3-DG–derived AGE formation at 37°C.** BSA (2 mg/ml) was incubated with (closed symbols) or without (open symbols) 200 mM 3-DG in PBS (pH 7.4) at 37°C for up to 28 d. The amounts of CML (A), 3-DG-imidazolone (B), pyrraline (C), and pentosidine (D) were quantified by non-competitive ELISA using 6D12 (0.1 μg/ml), JNH-27 (1 μg/ml), H-12 (10 μg/ml), and 1C12 (1 μg/ml). The immunoreactivity of each sample was determined at a fixed concentration of 3-DG-BSA (0.1 μg/ml).

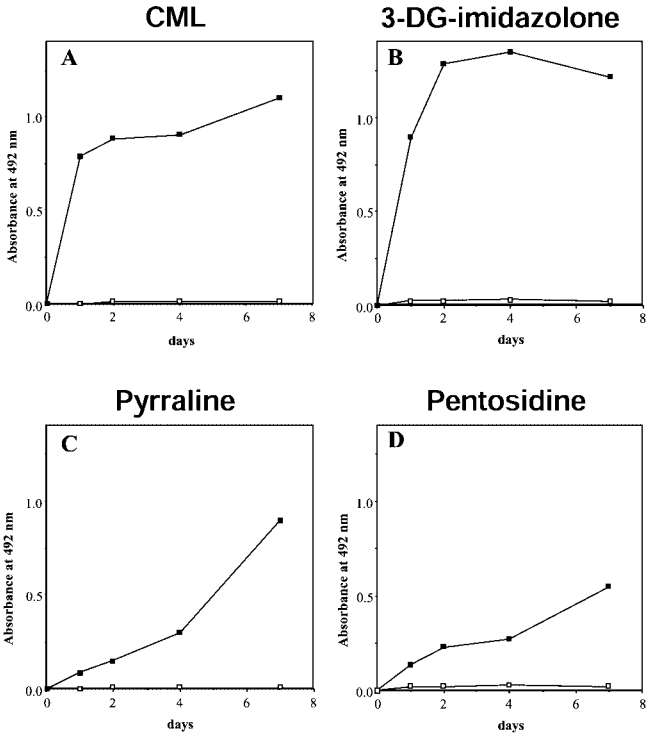

Fig. 4. **Time course of 3-DG–derived AGE formation at 60°C.** BSA (2 mg/ml) was incubated with (closed symbol) or without (open symbol) 200 mM 3-DG in PBS (pH 7.4) at 60°C for up to 28 d. The amounts of CML (A), 3-DG-imidazolone (B), pyrraline (C), and pentosidine (D) were quantified by non-competitive ELISA using 6D12 (0.1 μg/ml), JNH-27 (1 μg/ml), H-12 (10 μg/ml), and 1C12 (1 μg/ml). Immunoreactivity of each sample to these antibodies was determined at a fixed concentration of 3-DG-BSA (0.1 μg/ml).

tion, 3-DG was incubated with BSA at 37°C, and after various times, CML, 3-DG-imidazolone and pyrraline were measured by ELISA. As shown in Fig. 3A, 3-DG-BSA was recognized by the anti-CML antibody, and the extent of its reaction increased steeply with time. Likewise, the reactivity of 3-DG-BSA with the anti-3-DG–imidazolone antibody increased in a time-dependent manner (Fig. 3B). However, the reactivity of the anti-pyrraline antibody with 3-DG-BSA was very weak by day 14 and only increased slightly by day 28 (Fig. 3C). In contrast, the anti-pentosidine antibody did not show any reactivity with 3-DG-BSA even at day 28 (Fig. 3D). Since the formation of AGE increases at high temperatures (*20–22*), 3-DG-BSA was prepared under identical conditions except that the incubation was carried out at 60°C. The reactivity of both the anti-3-DG-imidazolone antibody (Fig. 4A) and the anti-CML antibody (Fig. 4B) with 3-DG-BSA, prepared at 60°C, reached a plateau level at day 2. Furthermore, the anti-pyrraline antibody (Fig. 4C) and the anti-pentosidine antibody (Fig. 4D) both showed significant reactivity in the day 1 sample, and their reactivities increased in a time-dependent fashion.

Next we also determined AGE contents in 3-DG-BSA incubated at 37°C for 4 wk by HPLC. When enzymatically hydrolyzed samples were subjected to HPLC as described in "MATERIALS AND METHODS," significant amounts of CML, 3-DG-imidazolone, pyrraline and pentosidine were observed (table 1). The CML content of 3-DG-BSA was 3.3- and 7.3-times higher than those of pyrraline and pentosidine, respectively (Table 1). These data suggest that CML and 3-DG-imidazolone are formed preferentially by 3-DG-modification compared to pyrraline.

Immunohistochemical Analysis of Atherosclerotic Lesions—For immunohistochemical analysis, we have compared four different stages of atherosclerosis. Samples of diffuse intimal thickening present a flat intimal thickening caused by increased fibrous tissue; fatty streaks show flat, slightly elevated lesions with local accumulation of foam cells; atheromatous plaques consist of elevated lesions with a central core of atheromatous debris covered by a fibrous cap; and complicated lesions are accompanied by superficial ulceration, thrombosis, and/or calci-

Table 1. **Determination of AGE structures in 3-DG-BSA by HPLC.**

AGE	Amount (mol/mol of BSA)
CML	1.10
3-DG-imidazolone	0.84
Pyrraline	0.33
Pentosidine	0.15

BSA (2 mg/ml) was incubated with 200 mM 3-DG at 37°C for 4 wk, and then dialyzed against PBS. AGE contents of the sample were quantified by HPLC after enzymatic digestion as described under "MATERIALS AND METHODS," and are expressed in mol/mol of BSA.

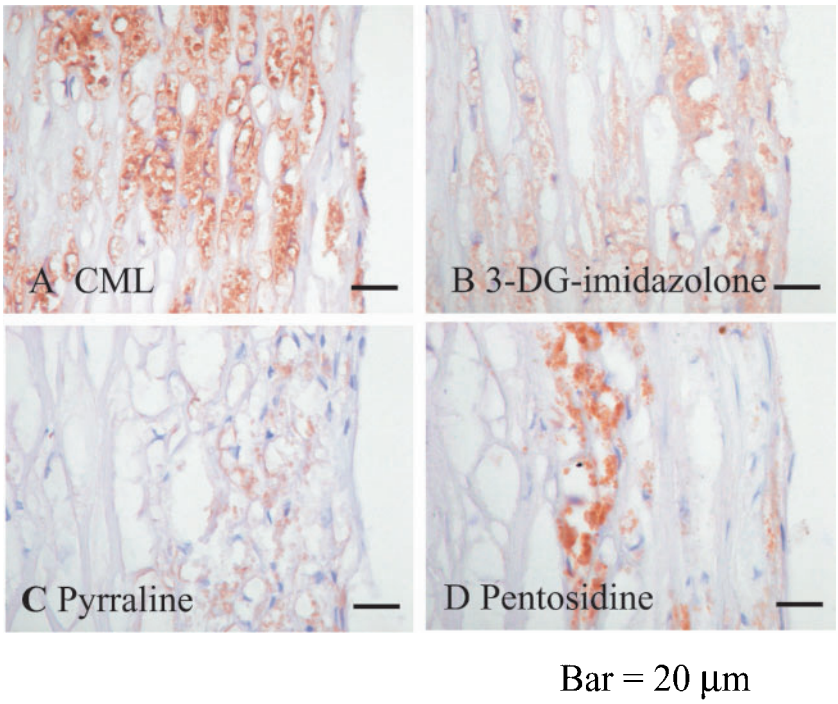

Fig. 5. **Immunohistochemical analysis of CML, 3-DG-imidazolone and pyrraline in intimal lesions of fatty streaks of the aorta.** The aorta was obtained from a 85-year-old male who died because of double cancers (adult T cell leukemia and lung carcinoma). Serial sections were stained with 6D12 (anti-CML antibody) (A), JNH27 (anti–3-DG-imidazolone antibody) (B), H-12 (anti-pyrraline antibody) (C), and 1C12 (anti-pentosidine antibody) (D). Magnification, × 270.

fication. Immunohistochemically, positive reactions to CML and 3-DG-imidazolone were demonstrated in the intimal lesions of fatty streaks, atheromatous plaques and complicated lesions, but were undetectable in diffuse intimal thickening as well as normal aorta. A strongly positive immunoreaction to CML (Fig. 5A) and moderately positive immunoreaction to 3-DG-imidazolone (Fig. 5B) were found intracellularly in the cytoplasm of most foam cells and extracellularly in the atheromatous core. A weakly positive reaction to pyrraline (Fig. 5C) was also found in the extracellular matrix and in a few foam cells in the intima of atherosclerotic lesions. Positive staining for pentosidine was noted intracellularly in the cytoplasm of most foam cells, but such immunostaining was not noted in the extracellular matrix (Fig. 5D). In the media and adventitia of aortas, no positive reactions for these AGE-structures were found.

DISCUSSION

Recent studies have demonstrated that several aldehydes including 3-DG (*6*), glycolaldehyde (*7*, *8*), glyoxal (*9*) and methylglyoxal (*6*) play important roles in the formation of AGE structures. In the present study, we analyzed 3-DG–modified BSA to demonstrate which AGE structures are preferentially generated during the modification of proteins by 3-DG. Our results showed that CML and 3-DG–imidazolone are the major AGE structures in 3-DG-BSA. Furthermore, our results suggest that 3-DG-imidazolone can be an important marker for the presence of 3-DG–modified proteins because CML is generated not only by protein modification by 3-DG but also by the cleavage of the Amadori product and Schiff base.

Hayase *et al*. (*31*) demonstrated that the generation of 3-DG in the Maillard reaction is accelerated under anaerobic conditions when lysozyme is incubated with glucose. In addition to the Maillard reaction, 3-DG is also generated from fructose through the polyol pathway especially in erythrocytes (*32*), and the hydrolysis of fructose-3-phosphate, which is generated by 3-phosphokinase (*33*). Niwa *et al*. (*34*) measured serum 3-DG levels in diabetic or uremic patients (*11*, *35*) by gas chromatography-mass spectrometry (GC-MS), and demonstrated the presence of high levels of 3-DG in their serum compared with normal subjects. Based on the GC-MS analysis, plasma 3-DG levels in normoglycemic subjects was 314 ng/ml and increased up to 778 ng/ml in diabetic patients (*34*). Yamada *et al*. (*36*) developed an HPLC method to determine 3-DG in plasma using 2,3-diaminonaphthalene, a dicarbonyl trapping reagent, and demonstrated that plasma 3-DG levels are elevated in streptozotocin-induced diabetic rats compared with normal rats. 3-DG itself shows certain biological activities such as the induction of apoptosis (*37*), suppression of cell proliferation and induction of heparin-binding of epidermal growth factor–like growth factor (*38*). Furthermore, 3-DG rapidly reacts with amino residues in proteins to form AGEs such as CML (*10*, *11*), 3-DG-imidazolone (*12*), pyrraline (*13*) and pentosidine (*14*). Taken together, these reports demonstrate that 3-DG plays an important role in protein modification *in vivo*.

In the present study, we have shown that the CML and 3-DG–imidazolone contents in 3-DG-BSA are higher than the pyrraline content (Fig. 3 and table 1). The 3DG-imidazolone content at day 7 was slightly lower than at day 4 (Fig. 4B), indicating that 3DG-imidazolone once formed may degrade during 7 d of incubation at 60°C because the structure is labile at high temperatures. Nagaraj *et al*. (*17*), however, reported the formation of 1,354 pmol of pyrraline/mg protein when bovine α-crystallins (100 mg/ml) were incubated with 100 mM 3-DG at 37°C for 19 d *in vitro*, and concluded that pyrraline is a

major 3-DG–derived AGE structure. In our system, 4,782 pmol of pyrraline/mg protein (0.33 mol of pyrraline/mol of BSA in table 1) was generated by incubating BSA (2 mg/ml) with 200 mM 3-DG at 37°C for 28 d, indicating that our pyrraline content in 3DG-BSA was 3.5-times higher than in the 3DG-α-crystallins determined by Nagaraj *et al*. (*17*). The difference between these results is regarded as a difference in the experimental system. Thus, the above group did not determine the CML and 3-DG-imidazolone contents in 3-DG–modified α-crystallins (*17*), whereas we detected four types of AGE, including CML, 3-DG-imidazolone, pyrraline and pentosidine, and confirmed that the pyrraline content in 3-DG-BSA was less than one-third that of CML (table 1). Our results are supported by the findings of Smith *et al*. (*18*) who confirmed that pyrraline is not an early-stage product or a major product in non-enzymatic protein glycation. Although pyrraline is proved to be a minor AGE structure in 3-DG–modified proteins (Table 1), it was detected in sclerosed glomeruli of kidneys of patients with diabetes (*16*), in sclerosed glomeruli of diabetic and aging individuals (*16*), senile plaques of patients with Alzheimer's disease (*5*), and the extracellular matrix in patients with atherosclerosis (*39*). Those observations suggest that pyrraline contributes to the development of each disease in its later stages or to the aging process. 1C12 failed to detect pentosidine in 3DG-BSA (Fig. 3D) even when pentosidine was detected in same sample by fluorescence HPLC. This lower sensitivity of pentosidine analysis by ELISA may be related to the nature of pentosidine molecule. Thus, since the pentosidine structure involves cross-links between lysine and arginine residues and may form intra- or intermolecular cross-links, the detection of pentosidine by antibody would be practically difficult if pentosidine is formed but hidden inside a protein molecule because of its cross-link property.

Since CML was proved to be a major AGE structure in 3-DG-BSA (Fig. 6), some portion of CML, which have been detected in several pathological conditions including diabetic nephropathy (*40*), atherosclerosis (*41*, *42*), hemodialysis-associated amyloidosis (*4*), chronic renal failure (*43*), hippocampal neurons (*44*), Pick's disease (*45*), peripheral nerves (*46*), atherosclerotic lesions in rat (*47*) aorta, and human skin elastin in actinic elastosis (*48*) may be generated by protein modification with 3-DG. However, CML is generated by several pathways, such as the oxidative cleavage of the Amadori product by hydroxyl radical (*49*), peroxynitrite (*50*) and Schiff base (*7*). In addition to these pathways, CML formation also takes place through glyoxal, which is generated from the autoxidation of glucose (*9*) and unsaturated fatty acids (*51*) (Fig. 6), indicating that 3-DG-imidazolone is more important as a potential marker for 3-DG–modified proteins than CML.

Previously, CML (*41*) and 3-DG-imidazolone (*15*) were independently reported to accumulate in macrophage/foam cells in atherosclerotic lesions. In this study, we measured these AGE structures simultaneously and found that both accumulate in similar areas of foamed macrophages in human atherosclerotic lesions. In contrast, a weakly positive immunoreaction to pyrraline was found in the extracellular matrix as well as intracellular lesions of a few foam cells. Regarding the difference in localization between CML, 3-DG-imidazolone and pyrraline, one hypothesis can be considered. Thus, several 3-DG–derived AGE structures are generated not only in the extracellular matrix but also in intracellular lesions. CML and 3-DG-imidazolone might be recognized by an AGE receptor expressed on cells such as macrophages and smooth muscle cells, and then taken up into the intracellular lesions. CML and 3-DG-imidazolone may both be recognized by the receptor for AGE (RAGE); it has been shown that CML-modified proteins are bound by RAGE (*52*) and that methylglyoxal-modified proteins containing almost exclusively methylglyoxal-derived hydroimidazolone bind to human monocytes via RAGE (*53*). It is conceivable that 3-DG–derived hydroimidazolone has similar activity. 3-DG modified proteins have also been found to bind the scavenger receptor type 1 (*54*). However, there is no report of an AGE receptor for pyrraline. Taken together, these results and reports strongly suggest that some of the CML and 3-DG-imidazolone generated in extracellular lesion were taken up through AGE receptor(s) into intracellular regions. Pyrraline might be generated slowly in both intra- and extracellular lesions. We also measured the localization of pentosidine in atherosclerosis lesions. Although the yield of pentosidine in 3-DG-BSA was lower than that of pyrraline (Table 1), a significant accumulation of pentosidine was observed in foam cells (Fig. 5D). Since pentosidine is generated not only from the Amadori product but also from short-chain aldehydes such as glyceraldehyde and glycolaldehyde (*55*), our results indicate that pentosidine might be generated in foam cells *via* glycolaldehydes generated from the myeloperoxidase system in activated macrophages (*56*). Taken together, our results provide the first evidence that CML and 3-DG-imidazolone are major AGE structures in 3-DG–modified proteins, and that 3-DG-imidazolone may be a better marker for 3-DG–modified proteins than pyrraline *in vivo*.

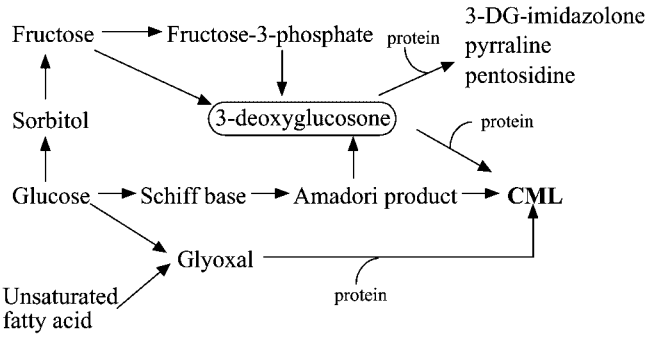

Fig. 6. **Possible pathways for 3-DG–derived AGE formation.**

We are grateful to Nahoko Nakano, Miki Cristina Hayashi, Kaori Takaki, Wakako Koito and Yuka Unno of our laboratory for their collaborative endeavors. This work was supported in part by Grants-in-Aid for Scientific Research (13470228 to Seikoh Horiuchi and 12770638 to Ryoji Nagai) from the Ministry of Education, Science, Sports and Culture of Japan.

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