Identification in Human Atherosclerotic Lesions of GA-pyridine, a Novel Structure Derived from Glycolaldehyde-modified Proteins*

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Glycolaldehyde (GA) is formed from serine by action of myeloperoxidase and reacts with proteins to form several products. Prominent among them is N\textsuperscript{-}(carboxymethyl)lysine (CML), which is also known as one of the advanced glycation end products. Because CML is formed from a wide range of precursors, we have attempted to identify unique structures characteristic of the reaction of GA with protein. To this end, monoclonal (GA5 and 1A12) and polyclonal (non-CML-GA) antibodies specific for GA-modified proteins were prepared. These antibodies specifically reacted with GA-modified and with hypochlorous acid-modified BSA, but not with BSA modified by other aldehydes, indicating that the epitope of these antibodies could be a specific marker for myeloperoxidase-induced protein modification. By HPLC, purification from GA-modified N\textsuperscript{-}(carboxymerloxy)-L-lysine, GA5-reactive compound was isolated, and its chemical structure was characterized as 3-hydroxy-4-hydroxyethyl-1-(5-amino-5-carboxypentyl) pyridinium cation. This compound named as GA-pyridine was recognized both by 1A12 and non-CML-GA, indicating that GA-pyridine is an important antigenic structure in GA-modified proteins. Immunohistochemical studies with GA5 demonstrated the accumulation of GA-pyridine in the cytoplasm of foam cells and extracellularly in the central region of atheroma in human atherosclerotic lesions. These results suggest that myeloperoxidase-mediated protein modification via GA may contribute to athrogenesis.

Modification of proteins with reactive aldehydes is thought to play a role in the pathogenesis of several diseases, including diabetes and atherosclerosis. Products of nonenzymatic glycation, Maillard reactions, such as 3-deoxyglucosone (1, 2), glyoxal (3), and methylglyoxal (4) are formed in tissue proteins in vivo and are considered to be precursors of advanced glycation end products (AGE).\textsuperscript{1} In a parallel pathway involving both enzymatic and nonenzymatic reactions during inflammation, leukocytes are activated to secrete myeloperoxidase, which mediates the formation of hypochlorous acid (HOCl) from hydrogen peroxide and chloride (5). Aldehydes such as GA, which is formed by reaction of HOCl with serine, then react to form chemical modifications in proteins. Myeloperoxidase has been detected immunohistochemically in lipid-rich advanced atherosclerotic lesions, and the active myeloperoxidase was purified from atherosclerotic arteries (6). A subsequent study by Hazell et al. (7) demonstrated that human atherosclerotic lesions were positively stained by a monoclonal antibody against HOCl-modified proteins, suggesting the presence of an epitope(s) specific for HOCl-modified proteins. HOCl generated by the myeloperoxidase system was shown to react with L-threonine to generate acrolein via 2-hydroxypropenol, whereas the similar reaction with L-serine led to formation of glycolaldehyde (GA) (8). An immunohistochemical study demonstrated the accumulation of an acrolein-protein adduct(s) in macrophage-derived foam cells as well as thickened neointima of human arterial walls (9). However, since acrolein is also formed by a pathway of lipid peroxidation (10), acrolein-protein adducts are not specific for myeloperoxidase-induced protein modification in vivo.

GA is known to react with protein amino residues to give brown-colored cross-linked structures (11). A recent study demonstrated that the reaction of myeloperoxidase-derived GA with RNase led to N\textsuperscript{-}(carboxymethyl)lysine (CML), indicating that CML is formed in GA-modified protein (8). However, since CML is also formed by the Maillard reaction pathways, such as oxidative cleavage of Amadori product (12), Schiff base (13), and autoxidation of glucose via glyoxal (14), CML is not a specific marker for myeloperoxidase-induced protein modification in vivo. Therefore, in order to obtain a specific marker for myeloperoxidase-induced protein modification in vivo, it is requisite to identify a GA-protein adduct(s) other than CML. Demonstration of such a specific marker in atherosclerotic lesions would establish a role for the myeloperoxidase system in chemical modification of proteins during athrogenesis.

To this end, we first attempted to prepare monoclonal and polyclonal antibodies against GA-modified proteins, which recognized GA-derived structure(s) other than CML. These antibodies did not show any cross-reactivity to BSA modified with AGE-producing aldehydes such as glucose, glyoxal, methylglyoxal, and 3-deoxyglucosone, indicating that the epitope of these antibodies could be specific for GA-modified proteins. We then isolated the epitope for these antibodies and determined...
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**MATERIALS AND METHODS**

**Chemicals**

n-Glucose, fatty acid-free BSA (Fraction V), ovalbumin, GA, and methylglyoxal were purchased from Sigma, and 3-deoxyglucosone and EDTA were purchased from Dojindo Laboratories (Kumamoto, Japan). N-(Carboxymethyl)lysine (CBZ-Lys) and N’-(acetyl-L)-lysine (acetyl-Lys) were purchased from Sigma. N’-4-(Butyloxyacarbonyl)histidine (Boc-His) was purchased from Nossbachochem. Acetylarginine (acetyl-Arg) was purchased from Peptide Institute, Inc. (Osaka, Japan). Horseradish peroxidase-conjugated goat anti-mouse IgG antibody was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Microtitration plates (96-well, Nunc ImmunoPlate-maxisorp) were purchased from Nippon Inter Med (Tokyo, Japan). Glyoxal was obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were of the highest grade available from commercial sources.

**General Procedure**

UV spectra were recorded on a Beckman (Fullerton, CA) model DU 650 spectrometer. 1H (500 MHz), 13C (126 MHz), 1H-detected heteronuclear multiple quantum coherence, 1H-detected multiple bond heteronuclear multiple quantum coherence (HMBQC), and nuclear Overhauser effect spectra were measured with a Bruker (Karlsruhe, Germany) model ARX-500 spectrometer in D2O. Mass spectra were measured on an Applied Biosystems/MDS Sciex (Foster City, CA) high-performance time-of-flight mass spectrometer. 1H-detected 2D nuclear Overhauser effect heteronuclear single-quantum coherence (NOESY-HSQC) spectrum was collected with a Bruker (Karlsruhe, Germany) model NMR-500 spectrometer in D2O. Amino acid analysis was performed with a liquid chromatograph (Mightysil RP-18 GP, 250 × 4.6 mm) (GL Science, Tokyo) equipped with a UV monitor (UV-970 and FP-970; autosampler, 851-AS; controller, 802-SC; column oven, 860°C; solvent mixing module, HG-980) and a mass spectrometer (Jasco, Tokyo, Japan). GC/MS analysis was performed with a gas chromatograph (GC-14B, Shimadzu, Kyoto, Japan) coupled with a GC-MS system (QP 1000 EX, Shimadzu, Kyoto, Japan) and a 5% phenyl methyl silicone capillary column (15 m × 0.32 mm i.d., 0.25 μm film thickness). The peak was measured at 30°C for 1 min, then the temperature was increased to 290°C at 12°C/min. Identification of a Novel Glycolaldehyde-derived AGE

**Enzyme-linked Immunosorbent Assay (ELISA)**

ELISAs were performed as described previously (18). Briefly, for noncompetitive ELISAs, each well of a 96-well microwell plate was coated with 100 μl of the test sample in PBS, blocked with 0.5% gelatin in PBS, and washed three times with PBS containing 0.05% Tween 20 (washing buffer). The wells were incubated with 0.1 ml of the indicated concentration of the primary antibody dissolved in washing buffer for 1 h. The wells were then washed with washing buffer three times and reacted with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody (Kirkegaard & Perry Laboratories), followed by reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated by 1.0 M sulfuric acid, and the absorbance at 492 nm was read by a micro-ELISA plate reader. For competitive ELISAs, each well of a 96-well microwell plate was coated with 100 μl of the indicated concentration of antigen in PBS and incubated for 1 h. After washing, the wells were washed three times with washing buffer and then blocked with 0.5% gelatin in PBS for 1 h. After triplicate washing with the washing buffer, 75 μl of the test sample was added, and then the same volume of the antibody solution was added to each well and incubated for 1 h. The antibodies bound to wells were detected by horseradish peroxidase-conjugated anti-mouse IgG antibody.

**Purification of Epitope Molecule of GA5 (GA5-epitope)**

**Step One—CBZ-Lys (100 μm) was incubated at 100 °C for 5 min with 50 mM GA (GA-CBZ-Lys) in 0.5 mM phosphate buffer (pH 7.4).** For preparative HPLC (Hitachi, Tokyo) (LaChrom system, pump, L-7100; autoinjector, L-7480; autosampler, L-7250; detector, UV-970; column oven, L-7300), the reaction mixture of GA-CBZ-Lys was injected (0.1 ml/each) into the reverse phase column (Mightysil RP-18 GP, 250 × 20 mm, Kanto Chemicals Co., Inc., Tokyo), equilibrated in a solution of 2% acetonitrile, and eluted at 2.70% acetonitrile from 0 to 55 min and 70% acetonitrile from 55 to 110 min. Effluents were monitored for fluorescence of λ_{exc}=326/406 nm and separated into three fractions (Fraction 1, 17–35 min; Fraction 2, 41–61 min; Fraction 3, 64–98 min). All analyses were performed at 40 °C at a flow rate of 5 ml/min. This fractionation was repeated 20 times, and each fraction was lyophilized and reconstituted in 2 ml of H2O, and the aliquot of each fraction (100 μl) was subjected to competitive ELISA by GA5. A portion of Fraction 1 (5 μl) obtained from step one was further applied to HPLC (Jasco, Tokyo, Japan) (pump, PU-9202; detector, UV-970 and FP-970; autosampler, 551-AS; controller, 802-SC; column oven, 860°C; solvent mixing module, HG-980) using a reverse phase column (Inertsil ODS-3V, 250 × 4.6 mm) (GL Science, Tokyo, Japan) and eluted with a linear gradient of 10% acetonitrile in 0.3% trifluoroacetic acid to 50% acetonitrile in 0.3% trifluoroacetic acid from 0 to 40 min, 50% acetonitrile in 0.3% trifluoroacetic acid from 40 to 50 min at a flow rate of 0.8 ml/min at 25 °C. Effluents were monitored by both absorbance at 270 nm and fluorescence of λ_{exc}=326/406 nm and separated into 25 fractions.

**Step Three**—The immunoreactive fractions (Fractions 11–13, 400 μl each) obtained from step two were pooled and reapplied to the same HPLC system but eluted with 27% acetonitrile in 0.3% trifluoroacetic acid.
acid (isocratic A) with a flow rate of 0.8 ml/min at 25 °C and separated into eight fractions based on absorbance at 270 nm and fluorescence at λex/em = 326/406 nm. This fractionation was repeated three times, and the combined lyophilized fractions were reconstituted in 1 ml of H2O for determination of immunoreactivity by competitive ELISA.

**Extensive Purification of GA5-epitope**

5 g of GA-modified CBZ-Lys (GA-CBZ-Lys) was prepared by incubating 50 mM GA and 100 mM CBZ-Lys at 100 °C for 5 min in 0.5 M phosphate buffer and then subjected to reverse phase medium pressure liquid chromatography using a Develosil LOP-45S column (500 × 45 mm; Nomura Chemical, Japan) and eluted with a 20% acetonitrile in 0.3% trifluoroacetic acid at a flow rate of 3.0 ml/min at room temperature. Eluents were monitored by absorbance at 270 nm and separated into 200 fractions (15 ml/fraction). Fractions 96–100 contained the same products as observed in Fraction 5 of step three above; these fractions were pooled and evaporated, and the residue (370 mg) was subjected to preparative reverse phase HPLC with a reverse phase column (Inertsil ODS-3, 250 × 20 mm) and eluted with 27% acetonitrile in 0.3% trifluoroacetic acid with a flow rate of 8 ml/min at 25 °C, and finally the substance identical to Fraction 5 in step three above was isolated by HPLC with a reverse phase column (Inertsil ODS-3, 250 × 4.6 mm), eluted with 20% acetonitrile in 0.1% acetic acid at a flow rate of 0.8 ml/min at 25 °C, and monitored by absorbance at 270 nm. The pooled product was recovered as a colorless syrup (6.3 mg).

**Deletion of Benzyloxycarbonyl Group from GA5 Epitope**

The benzyloxycarbonyl group of the epitope molecule (9.6 mg) was hydrolyzed in 1 ml of 30% hydrobromic acid/acetic acid at room temperature for 1 h. The reaction mixture was evaporated and redissolved in 1 ml of 1 N NaOH for hydrolysis of the O-acetyl ester derivative of the product. The reaction mixture was neutralized with 1 N HCl and then separated by HPLC with a reverse phase column (Inertsil ODS-3, 250 × 10 mm), eluted with 5% acetonitrile in 0.3% trifluoroacetic acid with a flow rate of 2.0 ml/min at 25 °C, and monitored by absorbance at 270 nm. The epitope molecule eluted at a retention time of 15.2 min was mixed with 1 ml of 0.1 N HCl and was evaporated to yield a colorless syrup (6.3 mg).

**Conjugation of GA5 Epitope to BSA**

To conjugate the purified GA5-epitope with BSA, 1 mg of GA5-epitope or 1 mg of acetyl-Lys as a control was incubated at room temperature for 1 h with 0.5 mg of BSA in the presence of 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Pierce) in 0.5 M 2-mercaptoethanol and dialyzed against PBS at 4 °C for 5 days. Normal aorta was obtained from a fibrosis patient (1 female; age 32). The specimens were composed of grossly normal regions, fatty streaks, atherosclerotic plaques, or complicated lesions. For immunohistochemistry, the tissues were embedded in OCT compound (Sakura Fine Technical Co., Tokyo, Japan), frozen in liquid nitrogen, and stored at −80 °C until use. Sections were cut 5 μm thick by a cryostat (HM-500M; Microm, Walldorf, Germany) and mounted onto poly-L-lysine-coated slides.

**Immunohistochemistry**

For immunohistochemical analysis, cryostat sections were prepared and examined by the indirect immunoperoxidase method. Briefly, after inhibition of endogenous peroxidase activity (19), the sections were incubated with GA5, washed with PBS, and reacted with peroxidase-labeled anti-mouse Fab (1/2) (Amersham Biosciences) diluted 1:100 as the second antibody. After visualization with 3,3'-diaminobenzidine (Dainichi Chemical Co., Kumamoto, Japan), the sections were counterstained with hematoxylin and mounted with Malinol (Muto Chemical Company, Tokyo, Japan). For negative controls, the same procedures were performed, but the first antibody was omitted. Nonimmune mouse IgG was also used as a negative control, and the staining results showed no immunoreaction. For the immunoadsorption test, GA5 was preincubated with 1 mg/ml GA-BSA at 37 °C for 1 h and reacted with peroxidase-labeled anti-mouse F(ab′)2.

**RESULTS**

**Reactivity of Monoclonal and Polyclonal Antibodies Against GA-modified Proteins**

The immunoreactivities of monoclonal (GA5 and 1A12) and polyclonal (non-CML-GA) antibodies against GA-modified proteins were determined by noncompetitive ELISA. GA5 significantly reacted with GA-BSA and HOCl-Ser-BSA, whereas 3-deoxyglucosone-modified BSA, methylglyoxal-modified BSA, glyoxal-modified BSA, CML-BSA, AGE-modified BSA, and native BSA were not recognized by the antibody (Fig. 1A). Similar results were obtained by 1A12 and non-CML-GA. Thus, these antibodies significantly reacted with GA-BSA and HOCl-Ser-BSA, whereas reactions to 3-deoxyglucosone-modified BSA, methylglyoxal-modified BSA, glyoxal-modified BSA, CML-BSA, AGE-modified BSA, and native BSA were below detection level. In contrast, 6D12 whose epitope was CML, reacted significantly not only with GA-BSA and HOCl-Ser-BSA but also with 3-deoxyglucosone-modified BSA, methylglyoxal-modified BSA, glyoxal-modified BSA, CML-BSA, and AGE-modified BSA. We then determined whether the GA-modified proteins contained GA5-epitope. GA5 immunoreacted with only not GA-BSA but also GA-ovalbumin and GA-LDL, whereas the unmodified proteins were not recognized by the antibody (Fig. 1B). Suggesting that GA-modified proteins contained a common AGE structure recognized by GA5. The time course study of formation of GA5-epitope and CML during the incubation of BSA with GA in vitro was then determined by noncompetitive ELISA using GA5 and 6D12. The GA5-epitope became detectable after 2 h, and its level increased rapidly up to 8 h, exhibiting a saturating tendency (Fig. 1C), whereas the levels of CML as determined by the reaction to 6D12 increased slowly but progressively until 24 h (Fig. 1C). The amino acid important for the generation of GA5-epitope was identified. Basic amino acids such as lysine, histidine, and arginine were used because these amino acids were thought to be modified preferentially by aldehydes (15). These amino acids were incubated with GA, and their reactivity to GA5 was determined by competitive ELISA. As shown in Fig. 1D, the reactivity of GA5 to GA-BSA was significantly competed for by GA-acetyl-Lys, but not by GA-Boc-His, GA-acetyl-Arg, or their unmodified amino acid. GA-acetyl-Lys lost its inhibitory activity upon acid hydrolysis, suggesting that the GA5-epitope is derived from lysine and an acid-labile nature.

**Purification of GA5-epitope**

CBZ-Lys was used as a starting...
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**Fig. 1. Immunoreactivities of GA5, 1A12, non-CML-GA, and 6D12 to modified BSA and amino acids.** A, each well of a 96-well microtiter plate coated with 5 μg/ml of the indicated samples was reacted with GA5 (0.1 μg/ml), 1A12 (0.1 μg/ml), non-CML-GA (1 μg/ml), or 6D12 (0.5 μg/ml). The antibodies bound to wells were detected by horseradish peroxidase-conjugated anti-mouse IgG (for GA5, 1A12, and 6D12) or anti-rabbit IgG (for non-CML-GA) as described under “Materials and Methods.” B, each well coated with the indicated concentration of the test sample was reacted with GA5 (0.1 μg/ml). The antibodies bound to wells were detected by horseradish peroxidase-conjugated anti-mouse IgG. C, each tube contained 2 mg/ml BSA and 10 mM GA in 1 ml of PBS was incubated at 37 °C for up to 24 h, and the reaction was terminated by the addition of 100 mM l-lysine, followed by dialysis against PBS. The immunoreactivity of each sample to GA5 and 6D12 was determined at a fixed concentration of antigen (1 μg/ml). D, each well was coated with 0.1 ml of 10 ng/ml GA-BSA and blocked with 0.5% gelatin. The test sample (50 μl) was added to each well in the presence of 50 μl of GA5 (0.02 μg/ml), followed by incubation for 80 min. The antibodies bound to wells were detected in the same way as described above.

material to identify the GA5-epitope, because CBZ group could be detected by UV absorption at 270 nm. GA-CBZ-Lys prepared by incubation at 100 °C for 5 min showed a similar immunoreactivity to GA-acetyl-Lys prepared by incubation at 37 °C for 4 days (data not shown). GA-CBZ-Lys was applied to the preparative HPLC (first step of purification), and the effluent was monitored with the fluorescence spectrum with an excitation maximum at 326 nm and emission at 406 nm, which is characteristic for GA-CBZ-Lys. Several fluorescent peaks were separated by the first step purification (Fig. 2A). These peaks were roughly separated into three fractions (Figures 1–3) at the indicated time periods, and their reactivity to GA5 was determined. Upon competitive ELISA, Fraction 1 showed the strongest inhibitory effect on the immunoreactivity of GA5 to GA-BSA (data not shown), indicating that GA5-epitope occurs mainly in Fraction 1. Fraction 1 (5 mg) was next applied to HPLC with an Inertsil ODS-3V column (second step of purification), and the effluent was monitored by both fluorescence and UV absorbance (Fig. 2B). Eluted peaks were fractionated into 25 fractions. Among them Fraction 12 showed a significant inhibitory effect on the immunoreactivity of GA5 to GA-BSA. Since Fractions 11 and 13 also showed a weak inhibition, these fractions were combined with Fraction 12 and further purified by HPLC. Several fluorescent peaks were observed (data not shown), and two major UV peaks at a retention time of 12.4 and 14.0 min were detected (Fig. 2C). These eluted peaks were fractionated into eight fractions at the indicated time periods, followed by determination of their reactivity to GA5. The reactivity of GA5 to GA-BSA was significantly inhibited by Fraction 5 (Fig. 2D). Since Fraction 5 could not be detected by a fluorescence detector, GA5-epitope was not fluorescent. The product in Fraction 5 was characterized by 1H NMR and ESI-MS: 81.18–3.18 (2H, m), 1.55–1.69 (1H, m), 1.69–1.95 (1H, m), 4.04 (1H, dd, J = 4.6, 9.1 Hz), 4.27–4.42 (2H, m), 4.72 (1H, s), 4.98 (1H, d, J = 12.5 Hz), 5.02 (1H, d, J = 12.5 Hz), 7.21–7.36 (5H, m), 7.80 (1H, d, J = 6.0 Hz), 8.10 (1H, s), 8.19 (1H, d, J = 6.0 Hz); ESI-MS: m/z 389 [M + H]+. 1H NMR of the CBZ group on CBZ-Lys: 4.97 (1H, d, J = 12.5 Hz), 5.04 (1H, d, J = 12.5 Hz), 7.25–7.40 (5H, m). These results suggested that Fraction 5 contained a single compound containing an aromatic ring other than the CBZ group.

**Structural Analysis of GA5-epitope—**The CBZ group of GA5-epitope was removed by 30% hydrobromic acid/acetic acid, followed by structural analysis by 1H NMR, 13C NMR, and ESI-MS as described under “Materials and Methods.” 1H-NMR (D2O) δ 1.31–1.42 (1H, m), 1.42–1.53 (1H, m), 1.84–1.96 (2H, m), 1.96–2.05 (2H, m), 2.94 (1H, t, J = 6.3 Hz), 4.47 (2H, t, J = 7.3 Hz), 4.81 (2H, s), 7.89 (1H, d, J = 6.1 Hz), 8.20 (1H, d, J = 1.0 Hz), 8.30 (1H, dd, J = 1.0, 6.1 Hz); 13C-NMR (D2O) δ: 21.0, 29.3, 29.8, 53.1, 58.0, 60.7, 124.7, 129.9, 136.1, 147.7, 153.3, 172.7; ESI-MS: m/z 255 [M + H]+. Fig. 3B summarizes the signals of both 1H-NMR and 13C NMR spectra from deprotected GA5-epitope. The assignments of protons and carbons were based on the result of 1H-detected heteronuclear multiple quantum coherence, HMBC, and nuclear Overhauser effect experiments. For this molecule, a cationic empirical formula C12H19N2O4 was assigned, because it showed peaks at m/z 255 in positive ESI-MS; 13C-NMR spectra showed 12 carbons, respectively. Although the protons and carbons of the lysine moiety were retained in GA5-epitope, the downfield shift of
methylene protons on the δ-carbon (4.47 ppm, 2H), suggested that the ε-nitrogen atom of the lysine moiety was part of an aromatic heterocycle. The $^{13}$C-NMR spectrum of deprotected GA5-epitope showed five carbons in the aromatic carbon region. Therefore, the aromatic part of this molecule seemed to be pyridinium cation. In an HMBC experiment (Fig. 3A), cross-peaks between protons of the δ-carbon of lysine and aromatic carbons were observed (H-1’–C-2, H-1’–C-6, H-2–C-1’, and H-6-C-1’). This result shows that C-2 and C-6 were adjacent to the nitrogen atom in the pyridinium ring. Moreover, H-6 was coupled with H-5 by 6.1 Hz and with H-2 by 1.0 Hz, which allowed us to assign C-2(CH)–N-1(CH$_2$–C-6(CH)–C-5(CH). The residual two carbons of the pyridinium ring (C-3 and C-4) were further substituted. The residual part of deprotected GA5-epitope was C$_3$H$_4$O$_2$, possibly consisting of one hydroxymethyl group and one hydroxy group. In the HMBC experiment, the cross-peaks between methylene protons in hydroxymethyl group at 4.81 ppm and C-3, C-4, and C-5 were also observed. In addition, the presence of nuclear Overhauser effect between the methylene protons and H5 led us to the conclusion that the hydroxymethyl group was attached to C-4.

Considered together, the results of these structural analyses indicated that the structure of deprotected GA5-epitope was 3-hydroxy-4-hydroxymethyl-1-(5-amino-5-carboxypentyl) pyridinium cation (Fig. 3B). Therefore, GA5-epitope is also referred to hereafter as GA-pyridine.

**Reactivity of Purified GA-pyridine to GA5**—The reactivity of purified GA-pyridine to GA5 was tested by competitive ELISA. The immunoreaction of GA5 to GA-BSA was significantly inhibited by GA-pyridine, whereas CBZ-Lys had no effect (Fig. 4A), confirming that the epitope of GA5 is GA-pyridine. The reactivity of both 1A12 and non-CML-GA to GA-BSA was also inhibited by GA-pyridine in a dose-dependent manner (data not shown). Furthermore, GA-pyridine-conjugated BSA (GA-pyridine-BSA) exhibited a strong positive reaction to GA5 (Fig. 4B), 1A12, and non-CML-GA (data not shown), whereas acetyl-Lys-conjugated BSA (acetyl-Lys-BSA), a negative control, had no effect on these antibodies (Fig. 4B). These results, taken together, provided convincing evidence for the notion that GA-pyridine is the GA5-epitope.

**Immunohistochemical Distribution of GA-pyridine in Human Atherosclerotic Lesions**—The tissue samples obtained...
from human aorta showed four histopathological features of atherosclerosis: (i) diffuse intimal thickening caused by increased fibrous tissues, (ii) fatty streaks or flat or slightly elevated lesions with local accumulation of foam cells, (iii) atherosclerotic plaques consisting of elevated lesions with a central core of atheromatous debris covered by a fibrous cap, and (iv) complicated lesions where atherosclerotic plaques were accompanied by superficial ulceration, thrombosis, and calcification. Upon immunohistochemical analyses, a positive reactivity for GA5 was noted in the aortic intima with atherosclerotic lesions including fatty streaks, atherosclerotic plaques, or complicated lesions, but no such immunoreactivity was noted in aortas with diffuse intimal thickening or in normal regions. Positive staining for GA5 was noted intracellularly in the cytoplasm of most foam cells (Fig. 5, A and B) and extracellularly in the central atheroma of the atherosclerotic core (Fig. 5, A and C). Double immunohistochemical staining showed that GA5-positive cells also reacted with KP-1, a positive marker for macrophage (Fig. 5C), suggesting accumulation of GA-pyridine in macrophages. However, no positive reaction for GA5 was found in the media and adventitia of the aorta. Furthermore, no immunoreactivity of GA5 was detected in normal aorta (Fig. 5D). The positive staining patterns of GA5 to foam cells were significantly weakened when GA5 was pretreated with GA-pyridine-conjugated BSA (Fig. 5E) but not with unmodified BSA (Fig. 5F).

DISCUSSION

Myeloperoxidase was detected in foam cells (6) and HOCl-modified proteins was also detected in human atherosclerotic lesions (7). HOCl is known to generate reactive aldehydes such as acrolein and GA. Furthermore, GA is known to cause protein cross-linking and produce CML. However, acrolein and CML are not specific markers for myeloperoxidase-induced protein cross-linking. Therefore, GA5 was used to detect GA and to study the accumulation of GA-pyridine in atherosclerotic lesions. GA5-positive reactivity was noted in aortas with atherosclerotic lesions, suggesting that GA-pyridine is a marker for atherosclerosis. GA5-positive cells were also present in macrophages, indicating that GA-pyridine is produced by macrophages in atherosclerotic lesions. The immunoreactivity of GA5 was weakened when GA5 was pretreated with GA-pyridine-conjugated BSA, suggesting that GA-pyridine is indeed a marker for atherosclerosis.
modification because they are also generated from lipid peroxidation (10, 21) and cleavage of Amadori products in the Maillard reaction (12), respectively. Therefore, a specific marker is required to elucidate the contribution of the myeloperoxidase system to protein modification in vivo. The specific question addressed in the present study was whether a GA-derived AGE structure other than CML is also generated in vivo. Our two monoclonal antibodies, GA5 and 1A12, and a polyclonal antibody, non-CML-GA, significantly reacted with GA- and HOCl/Ser-modified BSA, whereas the reactivity of these antibodies to other modified BSAs was below the limit of detection (Fig. 1A).

Our analysis successfully identified a new compound from the reaction mixture of GA with CBZ-lysine, and we named it GA-pyridine. Immunochemical analysis revealed that GA-pyridine was recognized not only by GA5 but also by 1A12 and non-CML-GA, indicating that GA-pyridine is a major immunological epitope in proteins modified with GA. Furthermore, our immunohistochemical studies using GA5 showed the presence of GA-pyridine in the cytoplasm of foam cells and extracellularly in the central atheroma of human atherosclerotic core. Specifically, the positive immunoreactivity of this antibody to these human atherosclerotic lesions was significantly reduced by the pretreatment of GA5 with GA-pyridine-conjugated BSA (Fig. 5E) but not with native BSA (Fig. 5F), providing the solid immunological evidence for the presence of GA-pyridine in vivo. These results therefore strongly suggest that myeloperoxidase is involved in the progression of atherosclerosis by promoting protein modification with GA to form GA-pyridine in the vascular wall.

Scheme 1 depicts a likely mechanism for GA-pyridine formation. First, the addition of two molecules of GA to the ε-amino group of lysine and dehydration forms intermediate 1. The further addition of GA to intermediate 1 and subsequent dehydration yields GA-pyridine via intermediates 2 and 3. For CML formation, the addition of one molecule of GA to the ε-amino group of lysine and dehydration forms GA-alkylimine, a Schiff base adduct of the amine with GA. This compound undergoes a rearrangement reaction to form aldoamine, which is then subjected to oxidative conversion to CML (13). Alternatively, GA may first be oxidized to glyoxal and then reacted with the ε-amino group of lysine to form CML (13).}

AGE structures so far identified include pyrraline (22), pentosidine (23), CML (24), crosslines (25), imidazolone (26, 27), imidazolium salt cross-links methylglyoxal-lysine dimer (MOLD), and imidazolium salt cross-links glyoxal lysine dimer (GOLD) (28), N\(^{\text{carboxyethyl}}\)lysine (29), vespertlysine (30), argpyrimidine (31), MRX (32), carboxymethylarginine (33), GALA, and GOLA (34). In sharp contrast to these products, GA-pyridine contains a pyridinium structure specifically derived from GA. To our surprise, GA-pyridine was recognized not only by GA5 but also by 1A12 and by polyclonal non-CML-GA, strongly suggesting that GA-pyridine is an important immunological epitope in GA-modified proteins. GA-alkylimine (Schiff base adduct of GA with amine) is thought to generate from the cleavage of Schiff base by the Namiki pathway of the Maillard reaction (35) (13). However, since GA-pyridine was below the limit of detection in glucose-modified BSA (Fig. 1A), the formation of GA-pyridine derived from the Maillard reaction is negligible or unlikely to occur. Furthermore, Robison et al. (36) detected GA when the monolayer of cultured tracheobronchial epithelial cells of guinea pigs was subjected to a 1-h exposure to NO\(_2\) (1 and 5 ppm). However, since conventional
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NO₂ concentration in the air is 0.03 ppm, the exposure of aorta to 1 ppm NO₂ is obviously far from physiological conditions. Therefore, to our knowledge, myeloperoxidase is an exclusive pathway for the formation of GA, at least under physiological conditions. Taken together, these results strongly suggest that GA-pyridine is an important marker for protein modification by the myeloperoxidase system.

We reported previously the presence of CML in human atherosclerotic lesions, both extra- and intracellularly, and that the areas of extracellular deposition were broader than those of intracellular accumulation (37), whereas immunoreaction for polyclonal antibody against AGE except for CML (anti-non-CML antibody) was predominantly found in extracellular lesions in human atherosclerotic lesions (38). In sharp contrast, immunoreaction for GA-pyridine was predominantly found in extracellular lesions. It is likely, therefore, that GA generated intracellularly by the reaction of HOCl with L-serine is involved in protein modification by GA-pyridine in situ.

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