Glycolaldehyde, a Reactive Intermediate for Advanced Glycation End Products, Plays an Important Role in the Generation of an Active Ligand for the Macrophage Scavenger Receptor

Ryoji Nagai, Kenshi Matsumoto, Xia Ling, Hiroshi Suzuki, Tomohiro Araki, and Seikoh Horiuchi

Long-term incubation of proteins with glucose leads to the formation of advanced glycation end products (AGEs) that are recognized by AGE receptors. Glyoxal, glycolaldehyde (GA), and methylglyoxal are potential intermediates for the formation of AGE structures such as N^{ε}-(carboxymethyl)lysine (CML). We evaluated the contribution of these aldehydes to the formation of AGE structure(s), particularly the structure important for the receptor-mediated endocytic uptake of AGE proteins by macrophages. GA-modified bovine serum albumin (BSA), methylglyoxal-modified BSA (MG-BSA), and glyoxal-modified BSA (GO-BSA) were prepared, and their physicochemical, immunological, and biologic properties were compared with those of glucose-derived AGE-BSA. CML contents were high in GO-BSA and low in GA-modified BSA (GA-BSA) but did not exist in MG-BSA. The fluorescence patterns of GA-BSA and MG-BSA were similar to those of glucose-derived AGE-BSA but were weak in GO-BSA. Immunochemically, the antibody against non-CML structures of glucose-derived AGE-BSA reacted strongly with GA-BSA and weakly with GO-BSA but did not react with MG-BSA. The negative charge of these ligands increased to a similar extent. However, GA-BSA, but not MG-BSA or GO-BSA, underwent receptor-mediated endocytosis by the macrophage-derived cell line RAW 264.7, which was effectively inhibited by glucose-derived AGE-BSA, acetylated LDL, and oxidized LDL, which are well-known ligands for the macrophage type I and type II class A scavenger receptors (MSR-A). The endocytic uptake of GA-BSA by mouse peritoneal macrophages was also significant, but that by peritoneal macrophages from MSR-A–deficient mice was markedly reduced. Our results suggest that GA serves as an important intermediate for the generation of AGE structure(s) responsible for recognition by MSR-A.
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In the Maillard reaction, incubation of proteins with glucose leads, through the formation of early products such as Schiff base and Amadori products, to advanced glycation end products (AGEs), which are characterized by fluorescence, a brown color, and intra- or intermolecular cross-linking. Immunohistochemical studies using anti-AGE antibodies have detected AGE modifications in several pathological tissues. These include kidneys of patients with diabetic nephropathy (1) and chronic renal failure (2), atherosclerotic lesions of arterial walls (3), amyloid fibrils in hemodialysis-related amyloidosis (4,5), and actinic elastosis of the photo-aged skin (6). These findings suggest the potential involvement of AGE modification in the pathogenesis of age-related diseases.

Cellular interactions with AGE proteins are known to induce several biological responses, including both endocytic uptake and degradation and induction of cytokines and growth factors, which are likely linked to the development of diabetic vascular complications (7). These responses are thought to be mediated by AGE receptors, which include the receptor for AGE (RAGE) (8,9), galectin-3 (10), macrophage type I and type II class A scavenger receptors (MSR-A) (11,12), 80K-H (13,14), and oligosaccharyltransferase-48 (OST-48) (13,14). Our recent studies using Chinese hamster ovary (CHO)-scavenger receptor cells (11) as well as peritoneal macrophages obtained from MSR-A knockout mice (12) have shown that MSR-A play a major role, particularly in the endocytic degradation of AGE proteins by macrophages and macrophage-derived cells. Despite extensive studies, ligand structures recognized by these AGE receptors have been poorly characterized.

In the Maillard reaction, a large number of AGE structures are formed through a complex of reactions (15) in which reactive intermediates are formed by enolization, dehydration, cyclization, fragmentation, and oxidation reactions. As shown in Fig. 1, an aldehyde group of reducing sugars undergoes a nonenzymatic condensation reaction with an amino group of proteins to form a Schiff base and undergoes a subsequent rearrangement to form a more stable Amadori prod-

From the Department of Biochemistry (R.N., K.M., X.L., S.H.), Kumamoto University School of Medicine, Kumamoto; Chugai Pharmaceutical (H.S.), Shizuoka; and the Faculty of Agriculture (T.A.), Kyushu Tokai University, Kumamoto, Japan.

Address correspondence and reprint requests to Seikoh Horiuchi, MD, PhD, Department of Biochemistry, Kumamoto University of School of Medicine, Honjo, 2-2-1, Kumamoto 860, Japan. E-mail: horiuchi@gpo.kumamoto-u.ac.jp.

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acetyl-LDL, acetylated LDL; AGE, advanced glycation end product; BSA, bovine serum albumin; CEL, N^{ε}-(carboxyethyl)lysine; CHO, Chinese hamster ovary; CML, N^{ε}-(carboxymethyl)lysine; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GA, glycolaldehyde; GA-BSA, glycolaldehyde-modified BSA; GO-BSA, glyoxal-modified BSA; GOLD, glyoxal-lysine dimer; HRP, horseradish peroxidase; MG-BSA, methylglyoxal-modified BSA; MOLD, methylglyoxal-lysine dimer; MSR-A, macrophage type I and type II class A scavenger receptors; ox-LDL, oxidized LDL; PBS, phosphate-buffered saline; RAGE, receptor for AGE; TCA, trichloroacetic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

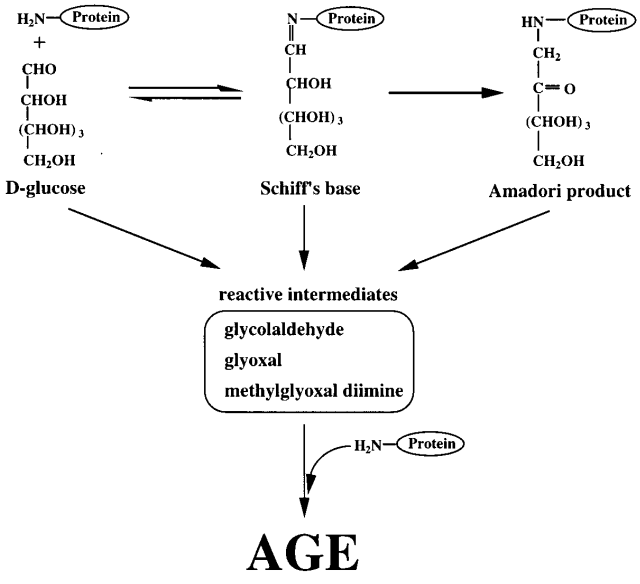

FIG. 1. GA, glyoxal, and methylglyoxal diimine as important intermediates for the formation of AGE structures in the Maillard reaction.

uct. The Schiff base may also undergo a retro-aldol reaction at C-2 → C-3 to yield glycolaldehyde (GA)-alkylimine (Schiff base adduct of GA with amine) (the Namiki pathway) (16,17). This compound may be hydrolyzed to release GA or rearranged to form an aldoamine (16,17). Aldoamine may react further with another amino group of the same or different protein to generate AGEs (18). Aldoamine may also be oxidatively converted to the glyoxal adduct, glyoxal-monoalkylimine, which is either converted to N^{ε}-(carboxymethyl)lysine (CML) (17) or forms a Schiff base cross-link by interaction with another amino group. The imidazolium salt cross-link, glyoxal-lysine dimer (GOLD) (19), is included in the glyoxal-derived AGE structure. Glyoxal generated directly through autoxidation of glucose (Wolff pathway) (20) also reacts with proteins to yield glyoxal-monoalkylimines such as CML and GOLD. On the other hand, Hayashi et al. (21) proposed that methylglyoxal diimine, a methylglyoxal protein adduct, is produced by cleavage of the Amadori product in the Maillard reaction. Methylglyoxal diimine may be converted to methylglyoxal-derived AGE structures, such as N^{ε}-(carboxyethyl)lysine (CEL) (22) and imidazolone (23), and to imidazolium salt cross-links, such as the methylglyoxal-lysine dimer (MOLD) (24). Therefore, it seems likely that GA, glyoxal, and methylglyoxal could contribute, as intermediates, to AGE structures generated in glucose-derived AGE proteins in vitro. Furthermore, in vivo presence of these aldehydes is also supported by data from several studies (19,24–27). For example, in addition to GA derived from the Maillard reaction, the myeloperoxidase pathway has been reported. GA (0.2 mmol/l) was generated when 0.2 mmol/l of L-serine was incubated with myeloperoxidase in the presence of hydrogen peroxide (25), indicating the possibility that GA is formed in vivo from L-serine by the myeloperoxidase-hydrogen peroxidase-chloride system of phagocytic leukocytes (28,29). Furthermore, another three studies describing AGE formation by GA were available. Anderson et al. (30) showed that CML, one of the major AGE structures in vivo, was generated in vitro when RNase was incubated for 90 min at 37°C with 60 mmol/l myeloperoxidase, 1 mmol/l L-serine, and 1 mmol/l hydrogen peroxide. CML was also generated when RNase was incubated with freshly harvested human neutrophils and 1 mmol/l L-serine at 37°C for 90 min (30). Daugherty et al. (31) showed that foam cells in human atherosclerotic lesions express a high myeloperoxidase activity, and a subsequent study by Schleicher et al. (32) demonstrated immunohistochemically the accumulation of CML in these foam cells. Determination of a free form of GA is practically impossible, due mainly to the high chemical reactivity of GA to amino residues of proteins. However, these reports suggest that proteins are modified by GA generated in vivo. In fact, Takeuchi et al. (33) demonstrated that two proteins of 200 and 0.85 kDa in human serum were shown by Western blot analyses to react with the antibody specific for GA-modified proteins (33). This is direct proof for the generation of GA in vivo. Although determination of a free form of GA derived from the Maillard reaction is practically impossible because of its high reactivity to amine (34), Glomb and Monnier (17) clearly demonstrated that 2,15-diamino-7,10-diaza-hexadecane-1,16-dioic acid, a reduced form of GA adduct with amine (C-2-imine), was generated during the Maillard reaction. In practice, 0.15 mmol of C-2-imine per mole of lysine was detected when bovine serum albumin (BSA) was incubated with 200 mmol/l ribose, a model Maillard reaction system (17). Therefore, proteins could be modified to a significant extent because of long-term exposure to GA in vivo.

In the present study, we compared these three aldehydes to determine their involvement in the formation of AGE structures. Specifically, AGE structure(s) important for the receptor-mediated endocytic uptake of AGE proteins by macrophages or macrophage-derived cells was analyzed. Results suggest that a GA-derived AGE structure(s) might, at least in part, be responsible for recognition by MSR-A.

RESEARCH DESIGN AND METHODS

Chemicals. GA, methylglyoxal, and fetal calf serum (FCS) were purchased from Sigma (St. Louis, MO). Our analyses with a Hantzsch reaction detected no formaldehyde in the methylglyoxal solution. Glyoxal (40% solution), D-glucose, BSA, and fatty acid–free BSA were purchased from Wako (Osaka, Japan). Tissue culture medium was from Gibco. Sodium ^{125}I was obtained from Amersham. All other chemicals were of the best grade available from commercial sources.

Modification with GA, methylglyoxal, and glyoxal. To prepare the GA-modified BSA (GA-BSA), methylglyoxal-modified BSA (MG-BSA), and glyoxal-modified BSA (GO-BSA), we incubated 2 mg/ml BSA (1.5 mmol/l lysine residues) with 33 mmol/l of GA, methylglyoxal, or glyoxal at 37°C for 7 days in phosphate-buffered saline (PBS) (pH 7.4). GA-BSA was also prepared from fatty acid–free BSA under identical conditions. Aliquots were obtained from each reaction mixture and dialyzed against PBS.

Preparation of CML-modified and CEL-modified BSA. CML-modified BSA was prepared as described previously (35). Briefly, 175 mg BSA was incubated at 37°C for 24 h with 0.15 mol/l glyoxylic acid and 0.45 mol/l $NaCNBH_3$ in 1 ml of 0.2 mol/l sodium phosphate buffer (pH 7.8), followed by dialysis against PBS. CEL-modified BSA was prepared from BSA as described previously (22,36). Briefly, 20 mg BSA was incubated at room temperature for 24 h with 1.8 mol/l sodium pyruvate and 0.3 mol/l $NaCNBH_3$ in 1 ml of 0.2 mol/l sodium phosphate buffer (pH 7.4), followed by dialysis against PBS.

Characterization of aldehyde-modified proteins. The modified BSA preparations were subjected to amino acid analysis after acid hydrolysis with 6 N HCl for 24 h at 110°C. Amino acids were analyzed with an amino acid analyzer (Model 835; Hitachi, Tokyo) using an ion exchange high-performance liquid chromatography column (number 2622 SC, 4.6 × 60 mm; Hitachi) and the ninhydrin postcolumn detecting system. CML contents were measured by amino acid analysis using hippuryl CML as the standard. The latter was pre-

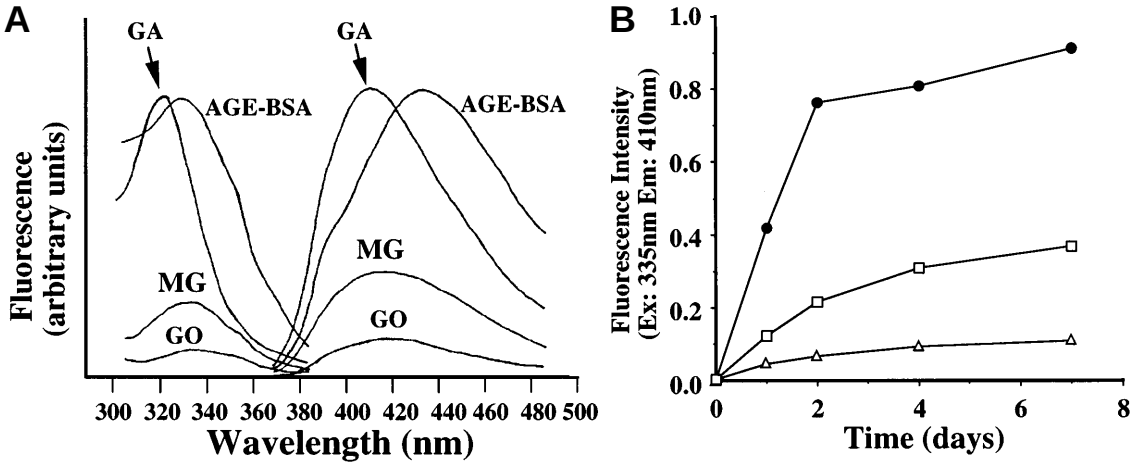

FIG. 2. Fluorescence measurements of GA-BSA, MG-BSA, and GO-BSA. *A*: The excitation (Ex) and emission (Em) fluorescence spectra were recorded at a constant emission wavelength (410 nm) and a constant excitation wavelength (335 nm), respectively, at a protein concentration of 67 μg/ml BSA derivatives. As a control, the fluorescent spectrum of AGE-BSA was measured at 20 mg/ml under the identical conditions. Samples used were GA-BSA, GO-BSA, and MG-BSA obtained by 7 days of incubation. AGE-BSA was prepared by 40 weeks of incubation as described in RESEARCH DESIGN AND METHODS. *B*: Serial changes in fluorescence intensity of aldehyde-modified proteins. Fluorescence intensity was measured at excitation (335 nm) and emission (410 nm) wavelengths. BSA (2.0 mg/ml) was incubated with 33 mmol/l glycolaldehyde (GA-BSA) (●), methylglyoxal (MG-BSA) (□), and glyoxal (GO-BSA) (△) at 37°C in PBS.

pared by incubating 40 mg/ml of hippuryllysine with 0.13 mol/l glyoxylic acid overnight at room temperature in the presence of 0.65 mol/l $NaCNBH_3$ in 0.5 ml of 0.1 mol/l sodium carbonate buffer (pH 10.0) (35,37). The extent of lysine modification of proteins was determined by reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (38) using unmodified BSA as the calibration standard. Briefly, 200 μl of 0.2 mol/l borate (pH 9.0) and 80 μl of 15 mmol/l TNBS were added to 200 μl of each sample. After incubation at 37°C for 30 min, the reaction was terminated by adding 800 μl 90% formic acid followed by measurement of absorbance at 420 nm. The arginine modification ratio was determined by 9,10-phenanthrenequinone (39,40). Briefly, modified BSA preparations in 50 mmol/l sodium borate (pH 8.5) were incubated at 37°C for 5 h with 37.5 μmol/l of 9,10-phenanthrenequinone and 0.25 mol/l sodium hydroxide. An equal volume of 1 mol/l hydrochloric acid was added to terminate the reaction, and fluorescence intensity (excitation, 312 nm; emission, 395 nm) was measured. The modification ratio of each sample was expressed as the percent of modification with unmodified BSA as a control. Agarose gel electrophoresis was performed using the Universal Gel/8 electrophoresis kit (Ciba-Corning, Tokyo) followed by staining with Coomassie brilliant blue (41).

Ligand preparation and iodination. Glucose-derived AGE-BSA was prepared as described previously (42). Briefly, 2.0 g BSA was dissolved in 10 ml of 0.5 mol/l sodium phosphate buffer (pH 7.4) with 3.0 g D-glucose, sterilized by ultrafiltration, and incubated at 37°C for 40 weeks followed by dialysis against PBS. Human LDL (d = 1.019–1.063 g/ml) was isolated from fasting human plasma by sequential ultracentrifugation from the plasma of normolipidemic subjects and dialyzed against 0.15 mol/l NaCl and 1 mmol/l EDTA (pH 7.4) (43). Acetylated LDL (acetyl-LDL) was prepared by chemical modification of LDL with acetic anhydride as described previously (44). To prepare oxidized LDL (ox-LDL), LDL was dialyzed against PBS to remove EDTA. LDL (0.1 mg/ml) was then incubated with 5 μmol/l $CuSO_4$ at 37°C for 24 h, followed by the addition of 1 mmol/l EDTA and cooling (45). To prepare maleylated BSA, 20 mg BSA in 10 ml of 0.1 mol/l sodium pyrophosphate buffer (pH 9.0) was added to 1 mol/l maleic anhydride in dioxane with a dropper on ice. The pH was continuously adjusted to 9.0 using NaOH solution. After incubation on ice for 5 min, the mixture was dialyzed extensively against PBS at 4°C (46). Modified BSA preparations were radiolabeled with ^{125}I using Iodo-Gen (Pierce) (47) and dialyzed against PBS.

Immunochemical quantification of non-CML structure by enzyme-linked immunosorbent assay. To detect AGE structures other than CML (non-CML structure), we used the rabbit polyclonal anti-AGE antibody for which the epitope was a non-CML structure as described previously (37). Briefly, from the polyclonal anti-AGE antibody (48), CML-specific antibodies were separated by affinity chromatography on a CML-modified BSA cellulofine column (37). Our recent immunochemical study demonstrated that the epitope of this antibody was generated in an oxidation-dependent manner (49). For the noncompetitive enzyme-linked immunosorbent assay (ELISA) (48), each well of a 96-well microtiter plate was coated with 100 μl of 1 μg/ml of GA-BSA, MG-BSA, or GO-BSA in 50 mmol/l sodium carbonate buffer (pH 9.6) and incubated for 1 h. The wells were washed three times with PBS containing 0.05% Tween 20 (washing buffer) and were blocked with 0.5% gelatin in 50 mmol/l sodium carbonate buffer (pH 9.6) for 1 h. After triplicate washing with the washing buffer, the wells were incubated for 1 h with 100 μl of the antibody (1 μg/ml). After triplicate washing with the washing buffer, the wells were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody, followed by a reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated with 1.0 mol/l sulfuric acid, and the absorbance was read at 492 nm on a microELISA plate reader. For competitive ELISA (48), each well of a 96-well microtiter plate was coated with 100 μl of 1.0 μg/ml of glucose-derived AGE-BSA in 50 mmol/l sodium carbonate buffer (pH 9.6) and incubated for 1 h. The wells were washed three times with washing buffer and then blocked with 0.5% gelatin in 50 mmol/l sodium carbonate buffer (pH 9.6) for 1 h. After triplicate washing with the washing buffer, 50 μl of the test sample and 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 HRP-conjugated anti-rabbit IgG antibody. For experiments using ELISA, the same set of experiments were repeated at least three times, and representative data are shown in Fig. 3*A* and *B*.

Cell culture. The murine macrophage–derived cell line RAW 264.7 was purchased from the American Type Culture Collection. Cells were cultured in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (medium A). Unless otherwise stated, cellular assays using RAW 264.7 cells and mouse peritoneal macrophages were performed at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cellular assays. For the binding study, 2.5×10^5 RAW 264.7 cells were seeded in each well of a 24-well culture plate (15.5-mm diameter; Corning) in 1.0 ml medium A and cultured for 12 h to subconfluence. The cells were washed with 1.0 ml PBS and replaced with ice-cold Dulbecco's modified Eagle's medium containing 3% BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin (medium B). The cells in each well were incubated at 4°C for 90 min in 0.5 ml medium B with various concentrations of ^{125}I-GA-BSA in the presence or absence of 100-fold unlabeled ligands. Each well was washed twice with 1.0 ml ice-cold PBS containing 1% BSA and two more times with ice-cold PBS. The cells were lysed with 1.0 ml of 0.1 N NaOH for 1 h at 37°C to determine the cell-bound radioactivity and cellular proteins. The protein concentration was measured by a bicinchoninic acid protein assay reagent (Pierce). The uptake study was carried out at 37°C under similar conditions. At the indicated time, 0.375 ml of the culture medium was taken from each well and mixed with 0.15 ml 40% trichloroacetic acid (TCA) in a vortex mixer. We added 0.1 ml of 0.7 mol/l $AgNO_3$ to this solution, which was followed by centrifugation. The resultant supernatant (0.25 ml) was used to determine TCA-soluble radioactivity, which was considered as an index of cellular degradation. The cell-associated radioactivity was determined as described for the binding study. Data represent the means ± SD of two separate experiments.

MSR-A gene knockout mice. Mice lacking both MSR-A were established from C57BL/6 by targeted disruption of exon 4 of the MSR-A gene, which is

essential for the formation of functional trimeric receptors in A3-1 ES cells, according to Suzuki et al. (12). Immunostaining of liver sections from wild-type mice using 2F8, a monoclonal antibody against MSR-A, detected MSR-A protein in sinusoidal cells. In homozygous MSR-A–deficient mice, no immunoreactive protein was detected (12), and cell culture experiments using peritoneal macrophages from these MSR-A knockout mice indicated a significantly low degradation for acetyl-LDL (18%) when compared with experiments from their wild-type littermates (12).

Mouse peritoneal macrophages. Peritoneal macrophages were collected from unstimulated male MSR-A gene knockout mice or wild-type littermates with 10 ml ice-cold PBS and centrifuged at 500*g* for 5 min. The cells were suspended at 2×10^6 cells/ml in medium B, and the cell suspension (1 ml) was dispersed into each well of a 12-well culture plate (22 mm in diameter; Corning) and then cultured for 18 h. The cell monolayers thus formed were washed three times with 1.0 ml PBS and used for the following experiments (11).

RESULTS

Physicochemical properties of aldehyde-modified proteins. We first compared the fluorescence properties of GA-BSA, MG-BSA, and GO-BSA with those of glucose-derived AGE-BSA. As shown in Fig. 2*A*, GA-BSA gave a fluorescent spectrum with an excitation maximum at 322 nm and emission maximum at 408 nm—a pattern identical to that reported for GA-RNase (18). MG-BSA showed a fluorescent spectrum with an excitation maximum at 335 nm and emission maximum at 415 nm. The excitation maximum of GO-BSA was 343 nm, and its emission maximum was 412 nm. The fluorescent patterns of GA-BSA, MG-BSA, and GO-BSA were similar to each other but slightly different from those of AGE-BSA, with an excitation maximum at 332 nm and an emission maximum at 429 nm (Fig. 2*A*). Their maximum fluorescence intensities increased with incubation time (Fig. 2*B*). The maximum fluorescent intensities of MG-BSA and GO-BSA were much weaker than that of GA-BSA (35 and 10% of GA-BSA, respectively).

In the next step, the aldehyde-modified BSA preparations obtained on days 2 and 7 were subjected to amino acid analysis. The results indicated that amino acid residues modified by these aldehydes were mainly basic amino acids such as lysine, histidine, and arginine. Table 1 summarizes the extent of lysine and arginine modification. The extent of lysine modification was 79% on day 2 and 83% on day 7 for GA-BSA, 39 and 46% for MG-BSA, and 37 and 38% for GO-BSA, whereas those of arginine modification were 20% on day 2 and 27% on day 7 for GA-BSA, 67 and 73% for MG-BSA, and 70 and 78% for GO-BSA. These data indicated that GA modifies lysine residues more selectively than arginine residues, whereas methylglyoxal and glyoxal reacted more favorably with arginine residues than lysine residues.

Table 1 also shows CML contents determined by amino acid analysis. CML contents of GA-BSA on days 2 and 5 were 1.0 and 1.2 mol/l of BSA, respectively, and those of GO-BSA were 14.6 and 14.4 mol/l of BSA, respectively. However, no significant CML was detected in MG-BSA. Thus, CML explained 2.5 and 64.0% of modified lysine residues in GA-BSA and GO-BSA on day 7, respectively, indicating that CML was the major modification product of GO-BSA. It is also clear from these data that CML formation in both GA-BSA and GO-BSA reached a plateau level as early as day 2. These results also demonstrated that fluorescence intensity highly correlates with the modification ratio of lysine residues but weakly correlates with the modification ratio of arginine residues.

We also determined the amount of AGE structures other than CML (non-CML structure) by using a polyclonal antibody specific for non-CML structure (34,49). Our previous studies have demonstrated that this antibody strongly reacts with AGE-modified proteins but not with known AGE structures such as pyrraline and pentosidine (37,48,49), and its epitope structure remains to be clarified. Noncompetitive ELISA showed that GA-BSA was recognized with this antibody, and the extent of its reaction steeply increased with time up to day 2. However, the reaction of this antibody to GO-BSA was

TABLE 1
Amounts of lysine, arginine, and CML in GA-BSA, MG-BSA, GO-BSA, and AGE-BSA

	Lysine			Arginine			CML	
	Residues	Modification (%)	TNBS method modification (%)	Residues	Modification (%)	Phenanthrenequinone method modification (%)	Residues	CML/modified lysine (%)
BSA	59.0	0	0	23.0	0	0	0	0
GA-BSA								
Day 2	12.2	79.4	72.1	18.3	20.3	26.6	1.0	2.1
Day 7	10.1	82.9	82.6	16.8	26.7	42.3	1.2	2.5
GA-BSA*								
Day 2	12.4	78.9	70.5	18.6	19.1	29.0	1.6	3.4
Day 7	10.0	83.0	75.1	17.8	22.6	44.6	2.0	4.1
MG-BSA								
Day 2	35.8	39.4	32.9	7.7	66.7	88.4	0	0
Day 7	31.9	45.9	42.2	6.1	73.3	81.4	0	0
GO-BSA								
Day 2	37.2	37.0	24.8	7.0	69.7	75.3	14.6	66.7
Day 7	36.5	38.1	28.6	5.1	77.9	83.1	14.4	64.0
AGE-BSA	14.3	75.7	95.2	7.7	66.5	93.1	1.0	2.2

Amounts of lysine, arginine, and CML in the modified BSA preparations were determined by amino acid analysis as described in RESEARCH DESIGN AND METHODS and expressed in residues per mole of BSA. Extent of lysine modification was also determined with TNBS, and that of arginine modification was determined by the 9,10-phenanthrenequinone method as described in RESEARCH DESIGN AND METHODS. *GA-BSA prepared from fatty acid–free BSA.

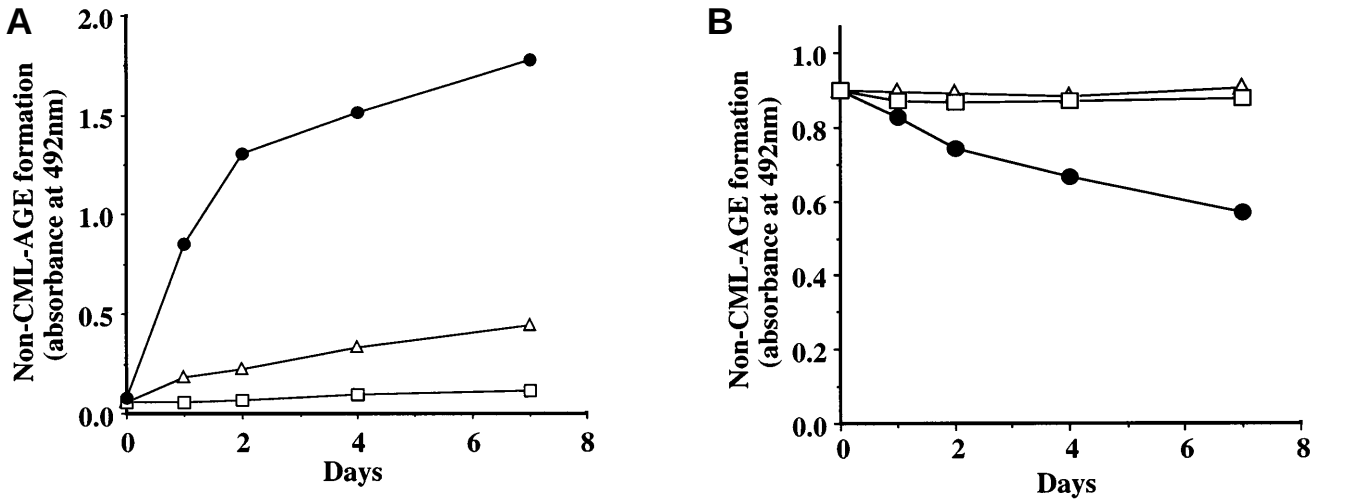

FIG. 3. Amount of non-CML AGE structure(s) determined by ELISA. For noncompetitive ELISA (*A*), each well of a 96-well microtiter plate was coated with 1 µg/ml GA-BSA, MG-BSA, or GO-BSA and incubated for 1 h. The wells were washed and blocked with gelatin, followed by reaction for 1 h with the antibody. The antibodies bound to wells were detected by HRP-conjugated anti-rabbit IgG antibody, as described in RESEARCH DESIGN AND METHODS. For competitive ELISA (*B*), each well coated with AGE-BSA was incubated with a mixture of 50 µl of test samples and 50 µl of the antibody solution, and antibodies bound to wells were detected by HRP-conjugated anti-rabbit IgG antibody, as described above. The tested samples were GA-BSA (●), MG-BSA (□), and GO-BSA (△).

very weak, and that to MG-BSA was almost negligible (Fig. 3*A*). The results obtained by competitive ELISA were consistent (Fig. 3*B*). The binding of this antibody to AGE-BSA was competitively inhibited by GA-BSA, and this inhibition increased with the incubation time. However, the binding was neither affected by MG-BSA nor GO-BSA.

Electrophoretic mobilities of these modified BSA preparations toward the anode were significantly higher than those of unmodified BSA; AGE-BSA migrated to a similar extent, although slightly slower than GA-BSA (Fig. 4). Under identical conditions, acetyl-LDL and ox-LDL, known ligands for MSR-A, showed a similar increase in their electrophoretic mobilities. Thus, it is likely that modification of BSA with these aldehydes is associated with a significant increase in the net negative charge.

Interaction of GA-BSA with RAW 264.7 cells. We next determined whether these aldehyde-modified BSA preparations could interact with cells as ligands. When 20 µg/ml of each ^{125}I-labeled ligand was incubated at 37°C for 18 h with RAW 264.7 cells, significant amounts of ^{125}I-GA-BSA were associated with these cells (Fig. 5*A*) and subjected to endocytic degradation by the same cells (Fig. 5*B*), whereas these changes were not observed in ^{125}I-MG-BSA and ^{125}I-GO-BSA (Fig. 5). Under these conditions, ^{125}I-AGE-BSA showed cell association and endocytic degradation equal to that of ^{125}I-GA-BSA. Thus, it is likely that only GA-BSA behaves as an active ligand for these cells. It is possible that fatty acids bound to BSA are oxidized to malondialdehyde during incubation with GA and affect the ligand activity of GA-BSA (50,51). We thus determined the ligand activity of GA-BSA prepared from fatty acid–free BSA under the identical assay conditions. The results showed that the cell association of GA (fatty acid–free) BSA to RAW 264.7 cells (1.4 µg/mg cell protein) and its capacity to be degraded by the cells (5.6 µg/mg cell protein) were indistinguishable from the results obtained above (Fig. 5*A* and 5*B*), indicating that the fatty acids bound to BSA

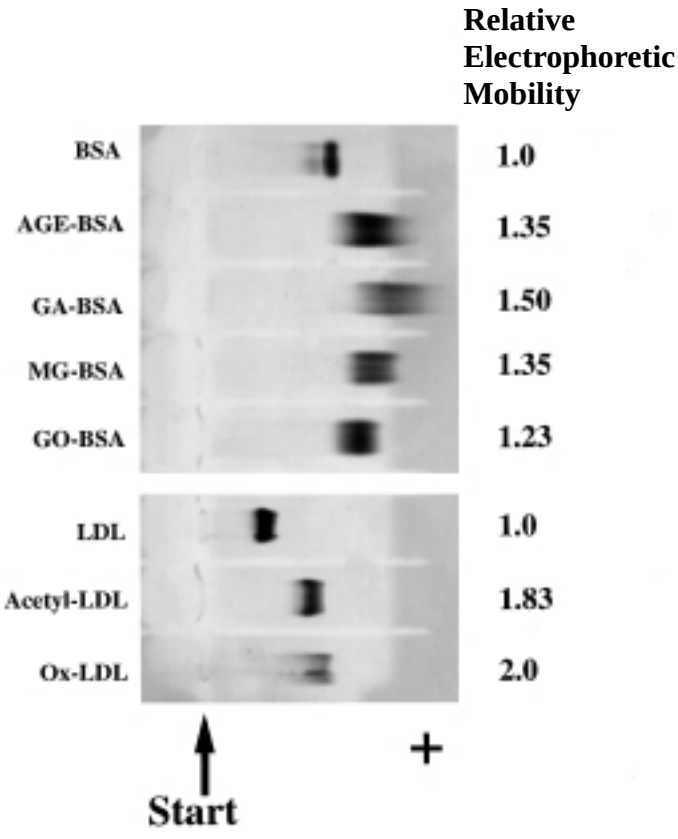

FIG. 4. Agarose gel electrophoresis of aldehyde-modified BSA and modified LDL. GA-BSA, MG-BSA, and GO-BSA prepared by 7 days of incubation and AGE-BSA were subjected to agarose gel electrophoresis and stained with Coomassie brilliant blue. Their electrophoretic mobilities relative to native BSA were expressed as relative electrophoretic mobility. Similarly, electrophoretic mobilities of modified LDL, such as acetyl-LDL and ox-LDL, were compared with those of native LDL.

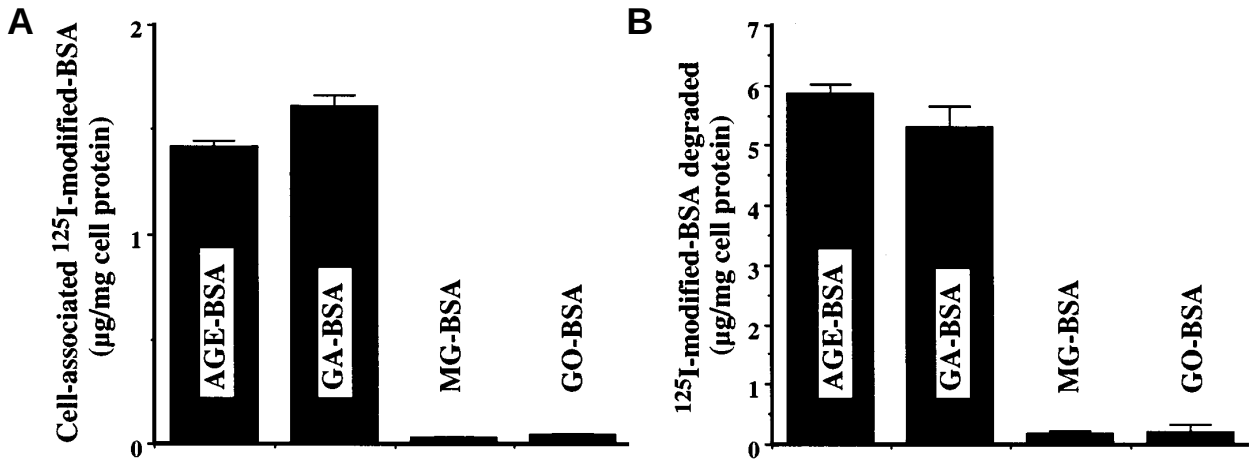

FIG. 5. Effects of aldehyde modification of BSA on endocytic degradation by RAW 264.7 cells. Cells were incubated at 37°C for 18 h with 0.5 ml of 20 μg/ml ^{125}I-AGE-BSA, ^{125}I-GA-BSA, ^{125}I-MG-BSA, or ^{125}I-GO-BSA. The amounts of cell-associated ligand (*A*) and degradation products (*B*) were determined as described in RESEARCH DESIGN AND METHODS. Specific cell association and degradation were calculated by correcting for nonspecific cell association and degradation. Data are means ± SD.

had no effect on the ligand activity. This notion was supported by the fact that amounts of lysine, arginine, and CML in GA (fatty acid–free) BSA did not differ from those in GA-BSA (Table 1). We also measured the ligand activity of GA (fatty acid–free) BSA for J774 cells, a murine macrophage–derived cell line. This ligand underwent effective degradation by these cells (7.3 μg/mg cell protein), indicating the high ligand activity of GA-modified proteins not only for RAW 264.7 cells but also for J774 cells.

Figure 6*A* shows the dose-dependent cellular binding of ^{125}I-GA-BSA at 4°C. Unlabeled GA-BSA was competitive for binding of ^{125}I-GA-BSA. The specific binding exhibited a saturation curve, and Scatchard analysis demonstrated a binding site with an apparent dissociation constant (K_d) value of 1.99 μg/ml and maximal surface binding of 0.13 μg/mg cell protein (Fig. 6*B*). The results indicated that RAW 264.7 cells possessed a high affinity binding site for GA-BSA. Incubation of these cells with ^{125}I-GA-BSA at 37°C increased the amount of cell-associated ^{125}I-GA-BSA, followed by secretion of TCA-soluble radioactivity into the medium (data not shown), indicating that GA-BSA undergoes receptor-mediated endocytosis with subsequent lysosomal degradation by these cells.

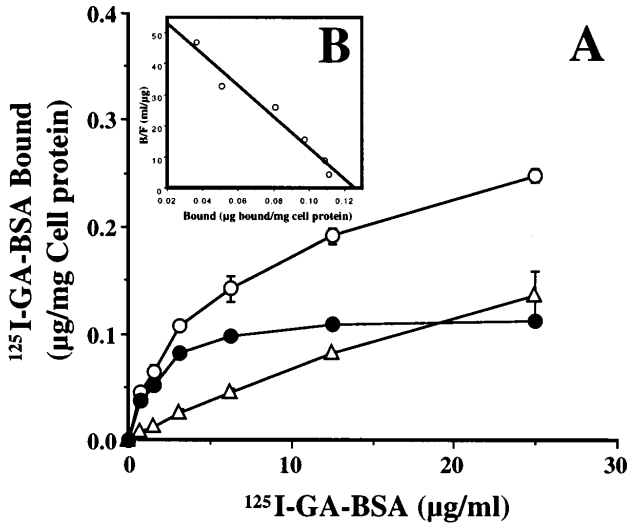

FIG. 6. Binding of ^{125}I-GA-BSA to RAW 264.7 cells. *A*: Cells in each well were incubated at 4°C for 90 min with the indicated concentration of ^{125}I-GA-BSA in the presence (△) or absence (○) of 100-fold unlabeled ligands. Cells were washed and lysed with 1.0 ml of 0.1 N NaOH for 1 h at 37°C to determine the cell-bound radioactivity and cellular proteins. Specific binding (●) was determined by subtracting nonspecific binding (△) from total binding (○). *B*: Scatchard analysis of the specific binding curve.

To further examine whether the recognition site for GA-BSA in RAW 264.7 cells was identical to that for AGE-BSA, we determined the cross-competitive effects of GA-BSA and AGE-BSA on their endocytic degradation. Degradation of ^{125}I-GA-BSA by these cells was effectively inhibited by both unlabeled GA-BSA and AGE-BSA (Fig. 7*A*). Similarly, endocytic degradation of ^{125}I-AGE-BSA was effectively inhibited by both unlabeled AGE-BSA and GA-BSA (Fig. 7*B*). Neither GO-BSA nor MG-BSA significantly inhibited the degradation of ^{125}I-GA-BSA or ^{125}I-AGE-BSA. Under these conditions, both CML-modified BSA (12.9 mol/l of BSA) and CEL-modified BSA (56.6 mol/l of BSA) also had no effect on the degradation of these ligands by RAW 264.7 cells (data not shown). Thus, the results of these cross-competition experiments are consistent with the notion that GA-BSA, but not GO-BSA or MG-BSA, is recognized by a receptor identical or closely similar to that for AGE-BSA in RAW 264.7 cells.

Biologic recognition of GA-BSA by the macrophage scavenger receptor. Five AGE receptors have so far been reported, including galectin-3 (10), RAGE (8), MSR-A (11), 80K-H (13,14), and OST-48 (13,14). Our recent experiments with CHO cells overexpressing type II MSR-A (11) and results of experiments using peritoneal macrophages obtained from MSR-A knockout mice (12) suggested a major role for MSR-A, at least in part, in the endocytic uptake of AGE proteins by macrophages or macrophage-derived cells. Based on these findings, we examined whether the receptor for GA-BSA in RAW 264.7 cells was identical to MSR-A. To this end, we tested the effects of ligands for the macrophage scavenger family, such as acetyl-LDL and ox-LDL, on the cel-

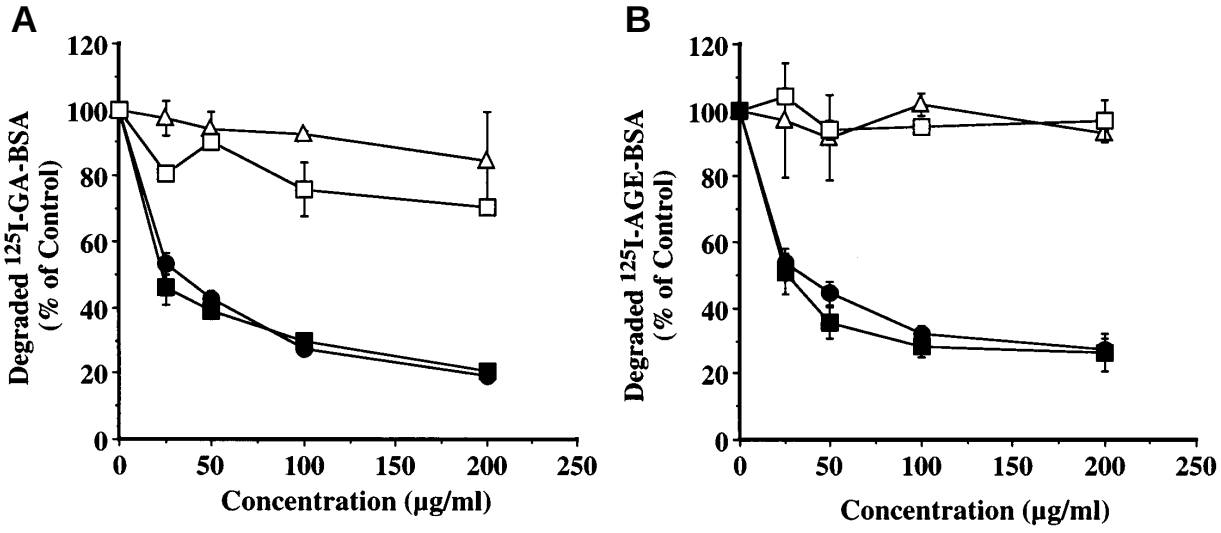

FIG. 7. Cross-competitive effects of GA-BSA and AGE-BSA on their degradation by RAW 264.7 cells. Cells were incubated at 37°C for 18 h with 0.5 ml of 2 μg/ml of ^{125}I-GA-BSA (*A*) or ^{125}I-AGE-BSA (*B*) in the presence of the indicated concentrations of unlabeled GA-BSA (●), AGE-BSA (■), MG-BSA (□), or GO-BSA (△). The values for 100% degradation of ^{125}I-GA-BSA (*A*) and ^{125}I-AGE-BSA (*B*) in the absence of unlabeled ligands were 1.07 and 0.79 μg/mg cell protein, respectively. Data are expressed as the percent of control and represent means ± SD of two separate experiments.

lular binding of ^{125}I-GA-BSA at 4°C. The cellular binding of ^{125}I-GA-BSA was effectively inhibited not only by unlabeled GA-BSA but also by AGE-BSA, acetyl-LDL, ox-LDL, and maleylated BSA, whereas neither GO-BSA nor MG-BSA showed any significant effect (Fig. 8*A*). Similarly, the cellular binding of ^{125}I-AGE-BSA was effectively inhibited by unlabeled GA-BSA, AGE-BSA, acetyl-LDL, or ox-LDL but not by GO-BSA or MG-BSA (Fig. 8*B*). It is possible therefore that GA-BSA is recognized by RAW 264.7 cells via one member of the scavenger receptor family.

To further confirm this notion, we compared the interaction of GA-BSA with peritoneal macrophages obtained from MSR-A knockout mice with that of wild-type macrophages. Peritoneal macrophages from wild-type littermates showed a significant endocytic capacity for ^{125}I-GA-BSA, whereas the corresponding endocytic degradation of ^{125}I-GA-BSA by peritoneal macrophages from MSR-A knockout mice was <20% of the wild-type macrophages (Fig. 9). Furthermore, whereas the endocytic degradation of ^{125}I-GA-BSA by wild-type macrophages was effectively competed for by unlabeled GA-BSA, acetyl-LDL, or AGE-BSA, the degradation of ^{125}I-GA-BSA by MSR-A knockout macrophages was totally not influenced by these unlabeled ligands (Fig. 9). These results indicate that MSR-A plays a major role in the endocytic degradation of GA-BSA by peritoneal macrophages.

DISCUSSION

Recent studies have suggested that several aldehydes, such as GA, glyoxal, and methylglyoxal, serve as important intermediates for the formation of AGE structures (17,19,22–24). The specific question addressed in the present study was which aldehyde among these potential intermediates was

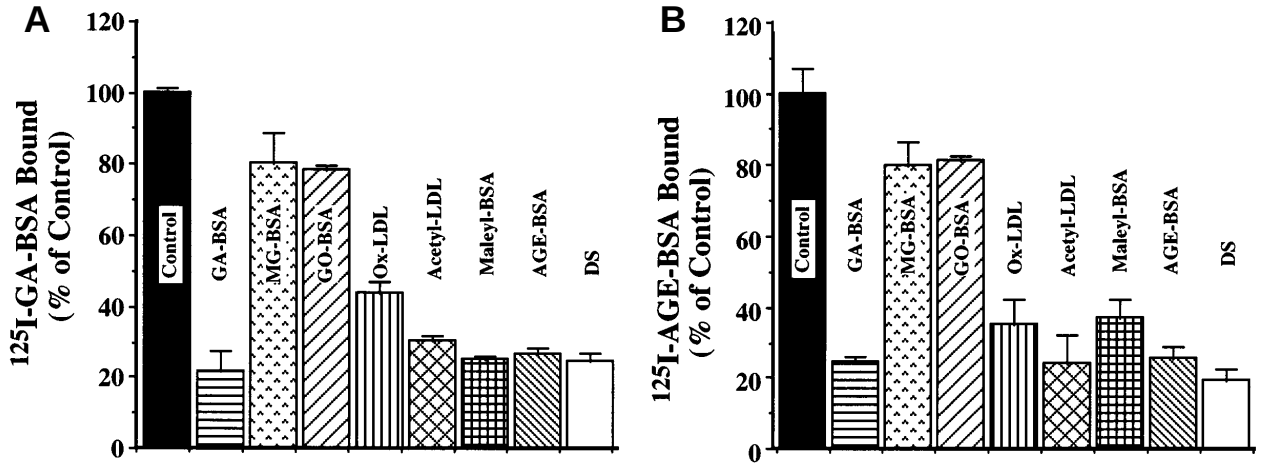

FIG. 8. Effects of several ligands on binding of ^{125}I-GA-BSA and ^{125}I-AGE-BSA to RAW 264.7 cells. The cells were incubated at 4°C for 90 min with 2 μg/ml of ^{125}I-GA-BSA (*A*) or ^{125}I-AGE-BSA (*B*) in the absence (control) or presence of 100-fold of unlabeled ligands: GO-BSA, MG-BSA, GA-BSA, ox-LDL, acetyl-LDL, maleylated BSA (maleyl-BSA), AGE-BSA, and dextran sulfate (DS). Amounts of cell-bound ^{125}I-GA-BSA were determined as described in RESEARCH DESIGN AND METHODS. Data are means ± SD.

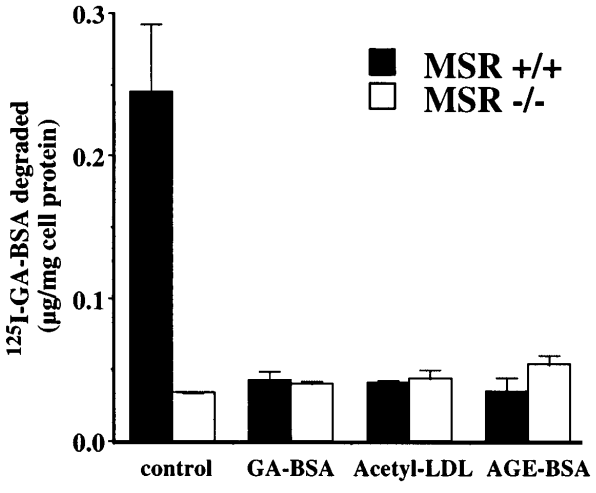

FIG. 9. Effect of GA-BSA on endocytic degradation by peritoneal macrophages from MSR-A knockout mice. Peritoneal macrophages (2×10^6/well) obtained from MSR-A knockout mice (□; $MSR^{-/-}$) and their littermates (■; $MSR^{+/+}$) were incubated for 18 h with 2 μg/ml GA-BSA in the absence (control) or presence of 100-fold of unlabeled ligands such as GA-BSA, acetyl-LDL, and AGE-BSA. Amounts of degradation products were determined as described in RESEARCH DESIGN AND METHODS. Specific degradation was calculated by correcting for nonspecific cell association and degradation. Data are means ± SD.

important for the generation of the AGE structure(s) recognized by AGE receptors. Taking this approach, the major findings of the present study were as follows. *1*) BSA modified by GA, but not by glyoxal or methylglyoxal, undergoes endocytic uptake and subsequent lysosomal degradation by murine macrophage–derived cell line RAW 264.7 cells. *2*) These processes were effectively inhibited by glucose-derived AGE-BSA as well as acetyl-LDL and ox-LDL, which are known ligands for MSR-A. *3*) Endocytic degradation of ^{125}I-GA-BSA by peritoneal macrophages from MSR-A knockout mice was markedly reduced compared with wild-type macrophages. These results strongly suggest that modification of BSA with GA but not with methylglyoxal or glyoxal might lead to the formation of an AGE structure(s) essential for the biologic recognition of AGE proteins by MSR-A.

Three major pathways have been proposed for CML formation (17,52). The first is derived from oxidative cleavage of Amadori adducts (53–55), the second is derived from Schiff bases (17), and the third is derived from glyoxal generated through autoxidation of glucose (20). In the present study, CML generated 2.5 and 64.0% of modified lysine residues in GA-BSA and GO-BSA, respectively. In sharp contrast, the amounts of the non-CML structure (AGE structure other than CML) in GA-BSA were markedly higher than those in GO-BSA or MG-BSA when determined by an antibody specific for non-CML structures (Fig. 3*A*). Further studies are needed to clarify the epitope structure of this antibody. Because CML does not undergo receptor recognition (56), it seems likely that non-CML structure(s) generated in GA-BSA may serve as a signal for the receptor recognition. Aldoamine, an Amadori-rearranged GA adduct, has been shown to form Schiff base linkages with another amino group, leading to covalent cross-linking of proteins (17,18). Therefore, chemical as well as immunological analyses of a structure(s) specific for GA-BSA could facilitate elucidation of the structure responsible for the recognition by MSR-A.

Amino acid analysis in the present study also showed that arginine residues were modified by glyoxal more preferentially than lysine residues (Table 1). Furthermore, glucose-derived AGE-BSA showed as high an extent of arginine modification as GO-BSA. Because glucose primarily reacts with lysine residues in the initial phase under antioxidative conditions, the high modification of arginine residues in glucose-derived AGE-BSA might be due to free glyoxal generated by autoxidation of glucose. In a manner similar to glyoxal, our results showed that arginine residues were preferentially modified by methylglyoxal (Table 1), a result consistent with those of Westwood et al. (57). Further studies are necessary to determine AGE structures derived from GA, glyoxal, or methylglyoxal. The present study emphasizes that among GA-BSA, GO-BSA, and MG-BSA, GA-BSA may play a major role as a recognition signal for MSR-A.

We previously showed that sinusoidal liver cells have a scavenger receptor for aldehyde-modified proteins such as proteins modified with aliphatic aldehydes (e.g., GA, DL-glyceraldehyde, and propionaldehyde), in which GA-BSA was most effective (58). The present data showing that both RAW 264.7 cells and mouse peritoneal macrophages recognize GA-BSA (but not GO-BSA or MG-BSA) are consistent with our early findings described above. MG-BSA has been shown, however, to undergo receptor-mediated endocytosis by murine P388D1 macrophages (57). No apparent difference was found between the MG-BSA preparation described by Westwood et al. (57) and our own preparation with regard to the physicochemical properties such as the extent of lysine and arginine modification and fluorescence spectrum (Table 1 and Fig. 2). These differences could reflect differences in the assay systems or cell types or both. For example, RAW 264.7 cells adherent to culture plates for 12 h (subconfluent) were subjected to an 18 h incubation assay in the present study, whereas the P388D1 cells in the above study were used in a suspension culture system for a 2-h incubation assay.

Although the present study showed that BSA modified with GA was recognized by MSR-A as an active ligand, the exact ligand structure(s) recognized by MSR-A is not clear. It is generally believed that the negative charge of the ligand is necessary; however, the ligand recognition by MSR-A is not explained solely by negative charges of the ligands (59). For instance, maleylation or succinylation of BSA results in an increase in the net negative charge, rendering it an effective ligand for MSR-A. On the other hand, whereas acetylation of BSA produces a similar increase in the net negative charge, such a change fails to generate the ligand activity for MSR-A (60). Moreover, our results also showed that modification of BSA with AGE structures, such as CML or CEL, increased the negative charge to an extent higher than glucose-derived AGE-BSA, but failed to generate an active ligand for MSR-A (data not shown). Therefore, the GA-derived structure(s) responsible for recognition by MSR-A could not be explained by a simple increase in the net negative charge of the ligands or the modification ratio of lysine residues. An alternative explanation would be a regional cluster(s) of the negative charges or a specific haptenic structure(s) of GA-BSA. The specific AGE structure(s) responsible for the receptor binding has not yet been identified, although a recent report by

Westwood et al. (61) provided evidence that N^{α}-acetyl-N^{δ}-(5-methyl-4-imidazolon-2-yl)-ornithine, a major AGE structure of methylglyoxal-modified BSA, behaves as a recognition factor for receptor binding in THP-1 cells. In addition, a recent report by Fu et al. (62) identified CML of AGE proteins as a ligand for RAGE. Further studies are needed to characterize the ligand structure for the AGE receptors.

Recent experiments using CHO cells overexpressing MSR-A (11) and peritoneal macrophages obtained from MSR-A knockout mice (12) clarified the important role of MSR-A in the endocytic degradation of AGE proteins by macrophages and macrophage-derived cells. Furthermore, immunological studies using anti-AGE monoclonal antibody (6D12) demonstrated extracellular AGE deposition as well as a dense accumulation of AGE intracellularly in macrophage-derived foam cells in early human atherosclerotic lesions (3). Because MSR-A is highly expressed on the foam cells of atherosclerotic lesions (63), these findings suggest that extracellular AGE proteins in the subendothelial space of arterial walls are endocytosed by monocyte-derived macrophages through MSR-A. Thus, to detect GA-modified proteins in vivo, we recently prepared a monoclonal antibody against GA-modified proteins (GA5). This antibody reacted positively with both GA-BSA and glucose-derived AGE-BSA but not with MG-BSA, GO-BSA, and 3-deoxyglucosone–modified BSA or known AGE structures, such as CML, CEL, pentosidine, methylglyoxal-derived hydroimidazolone, GOLD, MOLD, and pyrraline, suggesting that the epitope structure of GA5 may be related to a GA-derived AGE structure. In atherosclerotic lesions of human coronary arteries, GA5 reacted positively not only with foam cells, but also with extracellular matrix. However, reactivity of GA5 to atherosclerotic plaque competed with GA-BSA, indicating that GA5 specifically reacts with atherosclerotic plaque. These results indicate the presence of GA-derived AGE structures in these tissues. Furthermore, this pattern of immunohistochemical staining of human atherosclerotic lesions by GA5 resembled that obtained by staining with a monoclonal antibody against human MSR-A (MH1). Therefore, these findings strongly suggest that the GA-derived structures are present in monocytes/macrophage-derived foam cells in human atherosclerotic lesions in which MSR-A is highly expressed. Robison et al. (26) demonstrated that freshly harvested tracheobronchial epithelial (GPTE) cells from guinea pigs contained 20.3 nmol/l of GA, suggesting that GA, which was generated from GPTE cells, might be released into the blood in vivo. However, our attempt for determination of GA in vivo has been unsuccessful. It may due to the high chemical reactivity of GA to amino residues.

MSR-A mediate the endocytic uptake of chemically modified LDL, such as acetyl-LDL and ox-LDL (which result during the conversion of these macrophages into foam cells), as well as intracellular accumulation of cholesteryl esters (64). Therefore, LDL modified by GA in situ may also contribute to foam cell formation in atherosclerotic lesions.

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