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Glycolaldehyde-Modified Low Density Lipoprotein Leads Macrophages to Foam Cells *via* the Macrophage Scavenger Receptor[1]

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It was shown that proteins modified with advanced glycation end products (AGE) are effectively endocytosed by macrophages or macrophage-derived cells *in vitro*, and immunohistochemical studies involving anti-AGE antibodies demonstrated the accumulation of AGE-modified proteins (AGE-proteins) in macrophage-derived foam cells in human atherosclerotic lesions *in situ*, suggesting the involvement of AGE-modified LDL in the atherogenic process *in vivo*. To examine this suggestion, LDL was modified with glycolaldehyde, a highly reactive intermediate of the Maillard reaction. Physicochemically, glycolaldehyde-modified LDL (GA-LDL) was characterized by increases in negative charge, fluorescence intensity, and reactivity to anti-AGE antibodies, properties highly similar to those of AGE-proteins. The cellular interaction of GA-LDL with mouse peritoneal macrophages showed that GA-LDL was specifically recognized and endocytosed, followed by lysosomal degradation. The endocytic uptake of GA-LDL by these cells was competitively inhibited by acetylated LDL (acetyl-LDL), and the endocytic degradation of acetyl-LDL was also competed for by GA-LDL. Furthermore, incubation of GA-LDL with these macrophages and Chinese hamster ovary cells overexpressing the macrophage scavenger receptor (MSR), but not with peritoneal macrophages from MSR-knockout mice, led to the intracellular accumulation of cholesteryl esters (CE). These results raised the possibility that AGE-modified LDL, if available *in situ*, is taken up by macrophages mainly *via* MSR and then contributes to foam cell formation in early atherosclerotic lesions.

Key words: advanced glycation end products, atherosclerosis, foam cell formation, Maillard reaction, modified low density lipoprotein.

The early stages of atherosclerotic lesions are characterized by the presence of lipid-laden macrophage foam cells (*1*). Macrophages are known to take up chemically modified LDLs, such as acetylated LDL (acetyl-LDL) and oxidized LDL (Ox-LDL), *via* the macrophage scavenger receptor (MSR), leading to foam cell formation and massive intracellular accumulation of cholesteryl esters (CE) (*2*). Among the modified LDLs, Ox-LDL is regarded as a major atherogenic lipoprotein *in vivo*, due to its presence in human and rabbit atherosclerotic plaques (*3, 4*).

The incidence of atherosclerotic vascular disease is 3-4 times higher in diabetic patients than in normal subjects (*5*). As a cause of this higher incidence, it is generally accepted that hyperglycemia-induced glycation of plasma LDL results in a marked reduction in its ligand activity toward the LDL receptor, which leads to a delay of LDL clearance from the circulation and an increase in the plasma LDL cholesterol level, thus enhancing the atherogenic process in an indirect way (*6*). However, it was reported that hyperglycemia enhances the formation of advanced glycation end products (AGE) of the Maillard reaction. Moreover, recent immunohistochemical studies demonstrated AGE-accumulation in macrophage-derived foam cells in early atherosclerotic lesions (*7*). Furthermore, it recently became clear that AGE-modified proteins are taken up mainly by macrophages or macrophage-derived cells *via* MSR (*8, 9*). These findings suggest another interesting possibility that AGE-modified LDL *in situ* plays a direct role in the atherogenic process *in vivo*. To examine this notion, LDL was modified with glycolaldehyde, which is known to be a highly reactive intermediate of the Maillard reaction (*10*) as well as one generated from L-serine through the myeloperoxidase-hydrogen peroxidase-chloride system in activated neutrophils (*11*), and its physicochemical and biological properties were compared

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Abbreviations: AGE, advanced glycation end products; MSR, macrophage scavenger receptor; GA-LDL, glycolaldehyde-modified low density lipoprotein; AGE-LDL, AGE-modified LDL; acetyl-LDL, acetylated LDL; Ox-LDL, oxidized LDL; CE, cholesteryl ester; CHO cells, Chinese hamster ovary cells.

with those of AGE-BSA prepared by incubation with glucose.

MATERIALS AND METHODS

Reagents—Glycolaldehyde, BSA (Fraction V), mevalonic acid, fetal calf lipoprotein-deficient serum, and mevastatin (compactin) were purchased from Sigma (St. Louis, MO). Penicillin G (1,650 IU/mg), streptomycin sulfate (750 IU/mg), Dulbecco's modified Eagle's medium (DME), and Ham's F12 medium were purchased from Gibco. $Na^{125}I$ (17 Ci/mg) and [9,10-^{3}H]oleate (4 Ci/nmol) were purchased from Amersham International. Silica gel on aluminum sheets for TLC was obtained from Merk. Other chemicals were of the best grade available from commercial sources.

Lipoproteins and Their Modification—LDL (d=1.019-1.063 g/ml) was isolated by sequential ultracentrifugation from fresh human plasma from normolipidemic subjects and dialyzed against PBS containing 0.5 mM EDTA (pH 7.4). Carbon radicals are known to be produced through the autoxidation of glycolaldehyde and to participate in the lipid peroxidation of LDL (*12*). To prevent lipid peroxidation by carbon radicals, glycolaldehyde-modified LDL (GA-LDL) was prepared in the presence of EDTA. Briefly, 10 ml of LDL (2 mg/ml) was incubated at 37°C with 33 mM GA in PBS in the presence of 0.5 mM EDTA in the dark, and aliquots (2 ml) were collected at various intervals (4-96 h) and cooled on ice, followed by dialysis against PBS at 4°C. Acetyl-LDL was prepared by chemical modification of LDL with acetic anhydride as described previously (*13*). GA-LDL and acetyl-LDL were labeled with ^{125}I by the method involving Iodo-Gen (Bio-Rad) and by the procedure of McFarlane (*14*) to yield specific activities of 670 and 450 cpm/ng protein, respectively. Protein concentrations were determined with BCA protein assay reagent (Pierce Chemical) using BSA as a standard (*15*).

Physicochemical Analysis—The extents of chemical modification of lysine residues were determined as described previously (*16*) with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (*17*) using unmodified LDL as a calibration standard. Agarose gel electrophoresis was performed using a Universal Gel/8 electrophoresis system (Ciba-Corning Diagnostic, Tokyo), followed by staining with Coomassie Brilliant Blue (CBB) (*18*). SDS-PAGE was performed as described previously (*19*) with a modification. Each LDL preparation (10 μg) was electrophoresed on a 2-15% gradient polyacrylamide slab gel (Daiichi Pure Chemical, Tokyo), and then stained with CBB. The fluorescence spectrum of each GA-LDL preparation (150 μg/ml) was measured with a fluorescence spectrophotometer (Model 850; Hitachi, Tokyo), with excitation at 325 nm and emission at 400 nm (*20, 21*).

Immunochemical Analysis—ELISA was performed as described previously (*16, 22*). In brief, each well of a 96-well microtiter plate (6.4-mm in diameter, Nunc) was incubated for 1 h with 0.1 ml of a sample in 0.05 M carbonate buffer (pH 9.6), and then washed three times with PBS containing 0.05% Tween 20 (buffer A). Each well was blocked with 0.5% gelatin hydrolysate, washed with buffer A, and then reacted with 0.1 ml of buffer A containing 0.01 μg of a monoclonal or polyclonal anti-AGE antibody (*16*). The monoclonal antibody was specific for the N^{ϵ}-carboxymethyllysine (CML)-adduct, whereas the polyclonal antibody recognized a structure(s) other than that of CML. Each well was washed with buffer A and then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG or anti-rabbit IgG (Kirkegaard Perry Laboratories, Gaithersberg, USA), followed by reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated with 1 M sulfuric acid, and the absorbance at 492 nm was measured with a micro-ELISA plate reader (Titertek, Multiskan, Germany).

Peritoneal Macrophages from DDY Mice and MSR-Knockout Mice—Unless otherwise specified, all cellular experiments were performed at 37°C under a humidified atmosphere of 5% CO_2 in air. Peritoneal macrophages were collected from non-stimulated male DDY mice (25-30 g) in 8 ml of ice-cold PBS and then centrifuged at 500×g for 5 min. The cells were suspended at 2×10^6 cells/ml in DME containing 3% BSA, 100 units/ml of penicillin G, and 0.1 mg/ml of streptomycin sulfate (medium A). The cell suspension (1 ml) was dispersed into the wells of a 12-well culture plate (22-mm in diameter, Corning), and the cells were cultured for 2 h. The cell monolayers thus formed were washed three times with 1.0 ml of PBS and then used for the following experiments (*23*). Mice lacking both type I and II MSR were established from C57BL/6 by targeted disruption of exon 4 of the MSR gene in A3-1 ES cells according to the method described previously by Suzuki *et al.* (*9*). Peritoneal macrophages from these mice and wild-type littermates were collected and used for the experiments in the same way as described above.

MSR-Transfected CHO Cells—Chinese hamster ovary (CHO) cells overexpressing type II MSR (CHO-SR cells) were isolated according to the method of Freeman *et al.* (*24*). CHO-SR cells were selected and maintained in a stock medium ("MAC" medium) consisting of Ham's F12 supplemented with 5% fetal calf lipoprotein-deficient serum, 250 μM mevalonic acid, 3 μg/ml of acetyl-LDL, and 40 μM mevastatin (compactin) (*8*). The cells showed high activity as to the incorporation of DiI-labeled acetyl-LDL (*24*). CHO-SR cells (2×10^5 cells) were seeded into each well of a 12-well culture plate (22-mm in diameter; Corning), and then incubated for 16 h. The cell monolayers thus formed were washed three times with 1.0 ml of PBS and used for the following experiments (*8*).

Cellular Assay—For uptake studies, macrophage or CHO-SR cell monolayers were incubated for 5 h in 1.0 ml of medium A with various concentrations of ^{125}I-GA-LDL or ^{125}I-acetyl-LDL, with or without excess amounts of unlabeled ligands. An aliquot (0.75 ml) of the culture medium was taken from each well and mixed with 0.3 ml of 40% TCA on a vortex mixer. To this solution was added 0.2 ml of 0.7 M $AgNO_3$, followed by centrifugation. The resulting supernatant (0.5 ml) was used to determine the TCA-soluble radioactivity, which was taken as an index of cellular degradation (*8*). The remaining cells were washed twice with PBS containing 1% BSA and then twice with PBS. The cells were lysed at 37°C for 30 min in 1.0 ml of 0.1 M NaOH, and then the cell-associated radioactivity and cellular proteins were determined (*8, 13*). For the binding studies, macrophage or CHO-SR cell monolayers were incubated on ice for 2 h in 1.0 ml of medium A with various concentrations of ^{125}I-GA-LDL in the presence or absence of excess amounts of unlabeled ligands. Each well was washed twice with ice-cold PBS containing 1% BSA and

then twice with ice-cold PBS. The cells were lysed, and the cell-associated radioactivity was determined as described previously (*25*).

Assay for Cholesterol Esterification—[^{3}H]Oleate was conjugated with BSA as described previously (*26*). Macrophage monolayers from DDY mice, MSR-knockout mice, and their littermates (C57BL/6) (2×10^6 cells/well), or CHO-SR cells (2×10^5 cells/well) were incubated for 18 h in 1 ml of medium A with various concentrations of GA-LDL or acetyl-LDL in the presence of 0.1 mM [^{3}H]oleate (8.3×10^4 dpm/nmol). The cells were washed with 1 ml of PBS containing 0.3% BSA and twice with 1 ml of PBS, and then cellular lipids were extracted. The radioactive cholesteryl [^{3}H]oleate was determined by TLC (*27*). Cells were dissolved in 0.1 M NaOH and their protein concentrations were determined (*8, 13, 25*).

RESULTS

Physicochemical Properties of GA-LDL—As shown in Fig. 1A, the extents of lysine modification determined with the TNBS method increased sharply within 12 h (33% by 4-h incubation and 68.4% by 12-h incubation), followed by a gradual increase upon further incubation (from 80–90%), an almost plateau level being reached at 96 h. Figure 1B shows the electrophoretic patterns on agarose gel electrophoresis. Upon 4-h incubation with glycolaldehyde, LDL exhibited increased electrophoretic mobility and migrated as a slightly broadened band (Fig. 1B, lane 2). A further increase in modification was accompanied by an increase in the electrophoretic mobility, and the mobilities of GA-LDL preparations obtained with more than 24-h modification were almost the same as that of acetyl-LDL. It seems likely that the increase in the electrophoretic mobility reflects the lysine modification of LDL by glycolaldehyde. Glycolaldehyde-modified proteins are known to undergo cross-linking (*10*). As shown in Fig. 1C, all the GA-LDL preparations exhibited a ladder-like pattern of polymerization. The polymer band (approximately 1,000 kDa) seemed to correspond to a dimeric band of apolipoprotein B (apo B), indicating that apo B of LDL is polymerized on modification with glycolaldehyde.

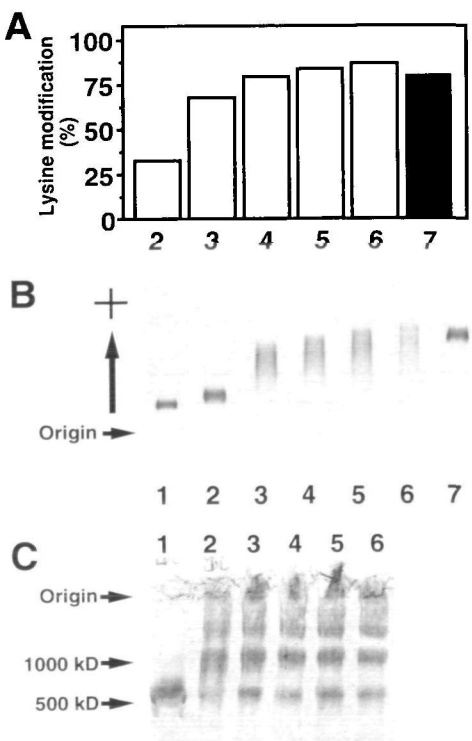

Fig. 1. **Physicochemical properties of GA-LDL.** (A) Determination of extents of lysine modification of GA-LDL preparations by the TNBS method. (B) Electrophoretic mobility of GA-LDL. GA-LDL preparations (2 μg/lane) were subjected to agarose electrophoresis, followed by staining with CBB. (C) Crosslink formation of GA-LDL. GA-LDL preparations (10 μg/lane) were electrophoresed on a 2-15% gradient SDS-PAGE plate and then stained with CBB. Lane 1, unmodified LDL; lanes 2-6, LDLs incubated with 33 mM glycolaldehyde for 4 h, 12 h, 24 h, 48 h, and 96 h, respectively; and lane 7, acetyl-LDL.

Figure 2 shows the fluorescence spectra of the GA-LDL preparations. Both unmodified LDL and acetyl-LDL were not fluorescent, whereas GA-LDL obtained with 4-h modification showed significant fluorescence with maximal excitation at 325 nm and maximal emission at 400 nm. The fluorescence pattern was closely similar or identical to previously reported for bone matrix proteins and RNase modified with glycolaldehyde (*20, 21*). The fluorescence intensities of these GA-LDL preparations increased with the extent of modification (Fig. 2). These results indicated that the physicochemical properties of GA-LDL, namely, lysine modification followed by increases in electrophoretic mobility, cross-linking and fluorescence activity, are highly similar to those of AGE-proteins (*28, 29*).

Immunochemical Properties of GA-LDL—To elucidate the immunochemical properties of GA-LDL, we examined the reactivity of these GA-LDL preparations with anti-AGE antibodies. The AGE-structures so far reported include fluorescent and cross-linked structures such as pentosidine (*30*), crosslines (*31*), and imidazolones (*32, 33*), and nonfluorescent and noncross-linked structures such as N^{ϵ}-carboxymethyllysine (CML) (*34*) and pyrraline (*35*). Two antibodies were used in the present study, one was specific for the CML-adduct (CML-specific antibody), and the other recognized a structure(s) other than that of CML, pyrraline, pentosidine and crosslines (non-CML specific antibody) (*16*). As shown in Fig. 3, ELISA with the

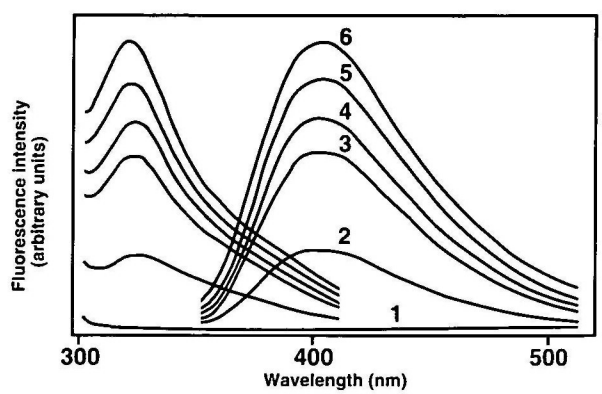

Fig. 2. **Fluorescence spectra of GA-LDL.** The fluorescence spectrum of each GA-LDL preparation was measured with a fluorescence spectrophotometer, with emission at 400 nm and excitation at 325 nm. The numbers on the fluorescence curves correspond to those in Fig. 1.

CML-specific antibody showed that CML of these GA-LDL preparations was detectable and their contents increased with the incubation time, indicating the formation of CML in these GA-LDL preparations as one of AGE-structures. Moreover, ELISA with the non-CML specific antibody also showed a positive reaction to these GA-LDL preparations, indicating that in addition to CML, a non-CML structure(s) of AGE was also formed during incubation of LDL with glycolaldehyde. These immunochemical results also supported the notion that LDL modified with glycolaldehyde shares properties with AGE-proteins.

Biological Properties of GA-LDL—As a biological property, AGE-proteins are known to be recognized and endocytosed actively by macrophages and macrophage-derived cells (*8, 9*). As a first step, we examined whether or not GA-LDL could bind to mouse peritoneal macrophages through a specific binding site. As shown in Fig. 4, the total binding of ^{125}I-GA-LDL to these cells increased dose-dependently, exhibiting a saturation pattern. The total binding of ^{125}I-GA-LDL was competed for by more than 80% by excess unlabeled GA-LDL. The specific binding, obtained by subtracting the non-specific binding from the total binding, also gave a similar saturation pattern. Scatchard analysis disclosed a high-affinity binding site on these cells' surface membranes with a K_d value of 2.1 μg/ml and maximum cell binding of 268 ng/mg cell protein (Fig. 4, inset), suggesting the presence of a specific GA-LDL binding site on these macrophages.

Experiments at 37°C were performed next to determine whether or not GA-LDL was endocytosed and degraded by mouse peritoneal macrophages in a receptor-mediated manner. During 5-h incubation, the amount of ^{125}I-GA-LDL degraded by these cells increased in a dose-dependent manner, and the degradation was effectively inhibited by more than 90% by an excess amount of the same unlabeled ligand (Fig. 5). The ligand concentration required for half saturation of the specific degradation was 3–4 μg/ml of GA-LDL, indicating that GA-LDL underwent receptor-mediated endocytosis, as in the case of modified LDLs such as acetyl-LDL and oxidized LDL (*36*). Under identical conditions, the amount of cell-associated ^{125}I-GA-LDL also

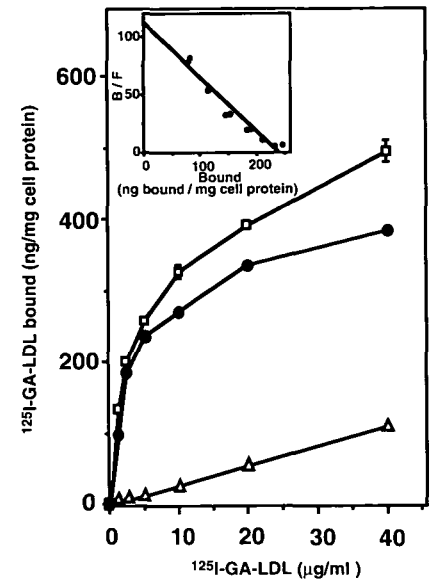

Fig. 4. **Cellular binding of ^{125}I-GA-LDL to mouse peritoneal macrophages.** Cells in monolayers were incubated for 90 min on ice with the indicated concentrations of ^{125}I-GA-LDL in the absence (□) or presence (△) of a 50-fold excess amount of unlabeled GA-LDL. The cell-bound radioactivity was determined as described under "MATERIALS AND METHODS." The specific binding (●) was obtained by subtracting the nonspecific binding from the total binding. Each bar denotes SD. The inset shows Scatchard analysis of the specific binding. The GA-LDL used was that incubated with 33 mM glycolaldehyde for 96 h, corresponding to lane 6 in Fig. 1.

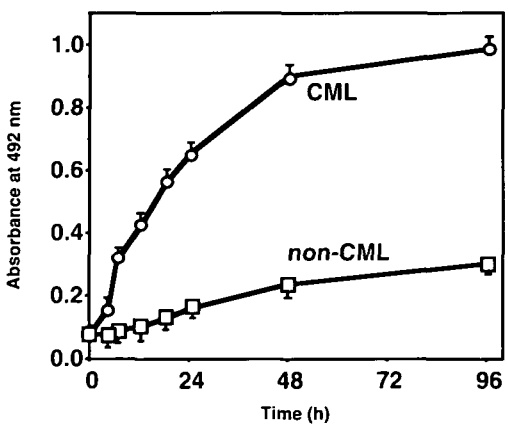

Fig. 3. **Immunochemical quantitation of CML and non-CML formation in GA-LDL.** The amounts of CML (○) and non-CML (□) in GA-LDL preparations were determined by the noncompetitive ELISA using the CML specific and non-CML specific antibodies as described under "MATERIALS AND METHODS." The immunoreactivity of each GA-LDL preparation to these antibodies was determined at a fixed GA-LDL concentration (0.1 μg/ml). The GA-LDL preparations used for this experiment were the same as those in Figs. 1 and 2.

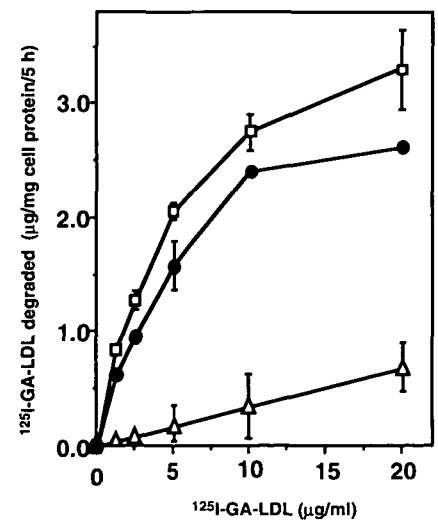

Fig. 5. **Endocytic degradation of ^{125}I-GA-LDL by mouse peritoneal macrophages.** Cells in monolayers were incubated for 5 h at 37°C with the indicated concentrations of ^{125}I-GA-LDL in the absence (□) or presence (△) of a 50-fold excess amount of unlabeled GA-LDL, followed by determination of the amount of ^{125}I-GA-LDL degraded as described under "MATERIALS AND METHODS." The specific degradation (●) was obtained by subtracting the nonspecific degradation from the total degradation. Each bar denotes SD. The GA-LDL used was the same as that in Fig. 4.

increased dose-dependently, and the increase was competitively inhibited by an excess of unlabeled GA-LDL (data not shown), supporting the notion that GA-LDL undergoes receptor-mediated endocytosis by these cells.

We next examined whether or not endocytic degradation of GA-LDL occurred through a route identical to acetyl-LDL, a well-known ligand for MSR. The endocytic degradation of ^{125}I-GA-LDL was inhibited dose-dependently by unlabeled acetyl-LDL (up to 200 μg/ml) in a manner closely similar or identical to GA-LDL, whereas LDL had no effect (Fig. 6A). Furthermore, the endocytic degradation of ^{125}I-acetyl-LDL by these cells was effectively competed for by either unlabeled GA-LDL or acetyl-LDL (Fig. 6B). From this cross-competitive effect of GA-LDL and acetyl-LDL, it is likely that MSR plays an important role in the endocytic degradation of GA-LDL by macrophages.

Modified LDLs such as acetyl-LDL and Ox-LDL are known to induce intracellular accumulation of cholesteryl esters (CE), leading macrophages to foam cells *in vitro* (*37*). As shown in Fig. 7A, GA-LDL preparations, particularly ones obtained with more than 12-h incubation, showed a marked capacity for CE accumulation. The GA-LDL obtained with more than 24-h modification showed a CE accumulation capacity similar to that of acetyl-LDL. We

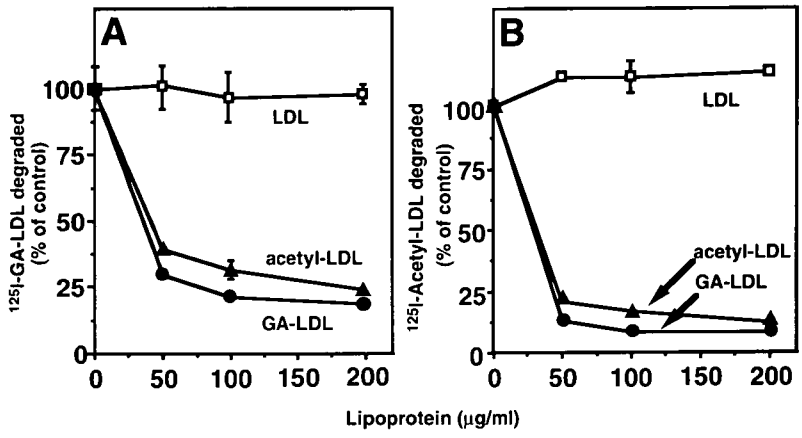

Fig. 6. **Cross-competitive effects of GA-LDL and acetyl-LDL on their degradation by mouse peritoneal macrophages.** (A) Cells in monolayers were incubated for 5 h at 37°C with 2 μg/ml of ^{125}I-GA-LDL in the presence of the indicated concentrations of unlabeled GA-LDL (●), acetyl-LDL (▲), or LDL (□). The amounts of ^{125}I-GA-LDL degraded were determined as described under "MATERIALS AND METHODS." The 100% value of degradation determined in the absence of unlabeled ligands was 1.13 μg/mg cell protein. Each bar represents SD. (B) Under identical conditions, cells were incubated for 5 h with 2 μg/ml of ^{125}I-acetyl-LDL in the presence of unlabeled GA-LDL (●), acetyl-LDL (▲), or LDL (□). The amounts of ^{125}I-acetyl-LDL degraded were determined in the same way. The 100% value of degradation was 0.70 μg/mg cell protein. Each bar denotes SD. The GA-LDL used was the same as that in Fig. 4.

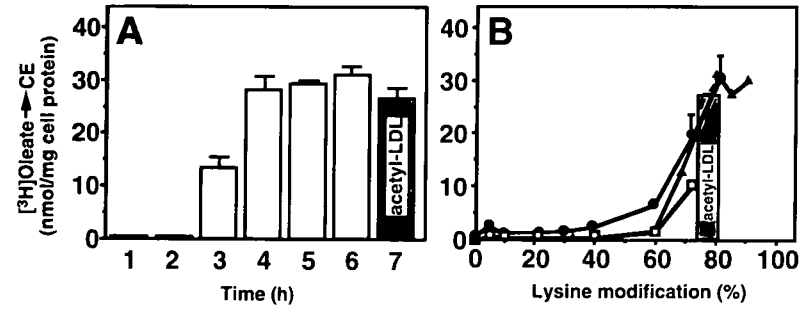

Fig. 7. **Effect of GA-LDL on CE accumulation in mouse peritoneal macrophages.** (A) Macrophage monolayers were incubated for 18 h with 10 μg/ml of different GA-LDL preparations or acetyl-LDL in the presence of 0.1 mM [^{3}H]oleate, and then cellular lipids were extracted, followed by determination of the radioactivity of cholesteryl [^{3}H]oleate as described under "MATERIALS AND METHODS." 1, unmodified LDL; 2-6, LDLs incubated with 33 mM glycolaldehyde for 4 h, 12 h, 24 h, 48 h, and 96 h, respectively; and 7, acetyl-LDL. (B) Correlation of the extents of lysine modification of GA-LDLs with their CE accumulation capacities. Three different batches of GA-LDLs were prepared by incubating LDL with 5-80 mM glycolaldehyde for 24 h and used for this experiment. The extents of lysine modification of GA-LDLs were 5-80% for the first batch (●), 33-90% for the second batch (▲), and 5-78% for the third batch (□). The extents of lysine modification of these GA-LDL preparations were plotted against their CE accumulating capacities. The closed bar shows acetyl-LDL with lysine modification of 78%. Each bar denotes SD.

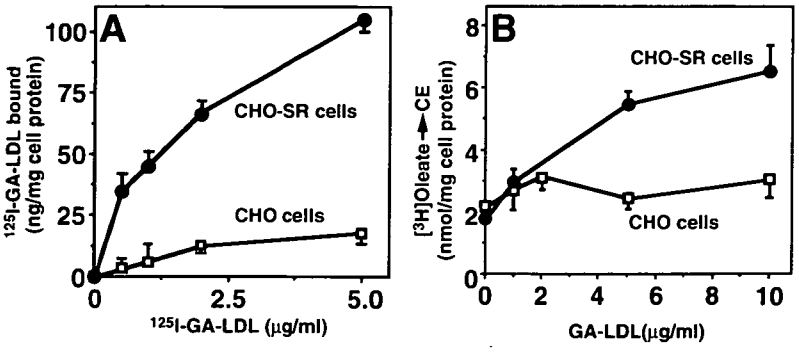

Fig. 8. **Interaction of GA-LDL with CHO-SR cells.** (A) Cellular binding of ^{125}I-GA-LDL to CHO-SR cells. CHO-SR (●) or CHO cells (□) were incubated for 90 min on ice with the indicated concentrations of ^{125}I-GA-LDL in the absence or presence of a 50-fold excess amount of unlabeled GA-LDL. The cell-bound radioactivity was determined as described under "MATERIALS AND METHODS." The specific binding was obtained by subtracting the nonspecific binding from the total binding. (B) Effect of GA-LDL on CE accumulation in CHO-SR cells. CHO-SR (●) or CHO cells (□) were incubated for 18 h at 37°C with the indicated concentrations (0 to 10 μg/ml) of GA-LDL in the presence of 0.1 mM [^{3}H]oleate. Cellular lipids were extracted, and the radioactivity of cholesteryl [^{3}H]oleate was determined as described under "MATERIALS AND METHODS." Each bar denotes SD. The GA-LDL used was the same as that in Fig. 4.

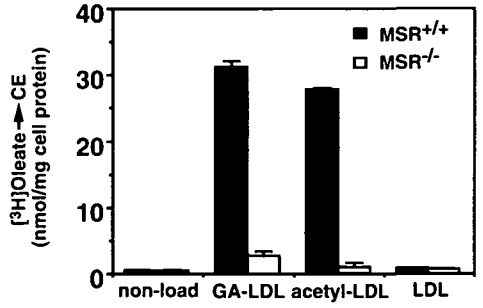

Fig. 9. **Effect of GA-LDL on CE accumulation in peritoneal macrophages from MSR-knockout mice.** Peritoneal macrophages (2×10^6/well) obtained from MSR knockout mice (□, $MSR^{-/-}$) and their littermates (■, $MSR^{+/+}$) were incubated for 18 h with 10 μg/ml of GA-LDL, acetyl-LDL, or LDL in the presence of 0.1 mM [^{3}H]-oleate as described under "MATERIALS AND METHODS." Cellular lipids were extracted and the radioactivity of cholesteryl [^{3}H]oleate was determined. Each bar denotes SD. The GA-LDL used was the same as that in Fig. 4.

next examined the correlation between the extent of lysine modification of GA-LDL and its CE accumulation capacity. For this purpose, we prepared GA-LDL samples with different lysine modifications. As shown in Fig. 7B, the CE accumulation capacities were negligibly weak for GA-LDL preparations whose lysine modification rates were lower than 50-60%. However, a further increase in the lysine modification converted GA-LDL into lipoproteins with a significant CE accumulation capacity.

To determine whether or not MSR was mainly involved in the GA-LDL-induced CE accumulation in macrophages, we performed binding experiments with ^{125}I-GA-LDL and CHO-SR cells which overexpressed type II MSR. Whereas the binding of ^{125}I-GA-LDL to wild-type CHO cells was negligible or extremely weak, the binding to CHO-SR cells was significant, dose-dependent and at least 5-fold higher than that in the case of wild-type CHO cells (Fig. 8A). We also examined GA-LDL-induced CE accumulation in CHO-SR cells. Although GA-LDL did not affect the CE content of wild-type CHO cells, the CE content of CHO-SR cells increased in a dose-dependent manner (Fig. 8B). Finally, we compared the CE-accumulation capacity of peritoneal macrophages prepared from MSR-knockout mice with that of wild-type littermates. The GA-LDL-induced CE accumulation in MSR-knockout macrophages was reduced to almost 20% of the wild-type level, under which acetyl-LDL-induced CE accumulation almost disappeared (Fig. 9). It is therefore evident that GA-LDL is endocytosed through a route identical to MSR, leading to foam cell formation.

DISCUSSION

Long-term incubation of proteins with glucose or reduced sugars leads, through the formation of early products such as a Schiff base and Amadori products, to AGE, compounds having unique properties such as fluorescence, browning and cross-linking (*28, 29*). Recent immunohistochemical studies involving anti-AGE antibodies have successfully demonstrated the presence of AGE in several human tissues, suggesting possible linking of AGE to aging (*19*), and age-enhanced disease states such as diabetic complications (*38-42*), atherosclerosis (*7, 20, 43, 44*), hemodialysis-related amyloidosis (*45, 46*), Alzheimer's disease (*47-50*), and actinic elastosis of the skin (*51*).

Incubation of LDL *in vitro* leads generally to oxidation and fragmentation of its apolipoprotein due largely to its high oxidizability. For the preparation of AGE-modified LDL, therefore, it would be desirable to modify LDL through short-term incubation with highly reactive intermediates of the Maillard reaction, instead of glucose (*10, 52*). A previous study indicated that the reaction of glycolaldehyde with proteins mimics the glycation of proteins with glucose (*21*). Therefore, LDL was modified with glycolaldehyde *in vitro* and used as a model AGE-modified LDL in the present study. Our GA-LDL preparations showed increases in relative electrophoretic mobility, cross-linking of apo B, and fluorescence intensity (see Figs. 1 and 2), and positive reactivity to two anti-AGE antibodies (*16*) (see Fig. 3), whereas the oxidizability of our GA-LDL preparations when determined as the TBARS level, was negligibly low as compared with that of conventional Cu^{2+}-modified Ox-LDL (data not shown). These results taken together indicate that GA-LDL shares a common structure(s) with AGE-proteins, supporting the notion that GA-LDL can be regarded as AGE-modified LDL physicochemically and immunochemically too.

Recent *in vitro* studies showed that a significant level of glycolaldehyde was in fact produced by cultured cells. Anderson *et al.* (*11*) indicated that activated neutrophils were able to generate 0.2 mM glycolaldehyde from 0.2 mM L-serine through the myeloperoxidase-hydrogen peroxidase-chloride system. The correspondence of this concentration to the plasma level of L-serine led these authors to propose the likely production of glycolaldehyde *in vivo*. Furthermore, Robinson *et al.* (*53*) reported that the intracellular concentration of glycolaldehyde reached 3 mM when tracheobronchial epithelial cells of guinea pigs were exposed to nitrogen dioxide for 1 h. Although the concentration of glycolaldehyde used to prepare GA-LDL in the present study (33 mM) seemed to be far from its physiological level, these *in vitro* experiments revealed the possibility that glycolaldehyde is generated in some pathological conditions. Even though its concentration generated *in vivo* is extremely low, proteins accumulated or deposited in the extracellular space could be modified to a significant extent due to long-time exposure to glycolaldehyde. This is one of the major issues as to the pathophysiological relevance of protein modification by glycolaldehyde or AGE.

It is a general belief that AGE-proteins undergo specific recognition by cell surface receptors such as the receptors for AGE (RAGE) (*54, 55*), galectin-3 (p90) (*56*), and MSR (*8, 9*). Our recent studies involving CHO-SR cells (*8*) and peritoneal macrophages obtained from MSR-knockout mice (*9*) clearly indicated a major role of MSR in the endocytic degradation of AGE-proteins by macrophages and macrophage-derived cells. From its ligand specificity as well as its interaction with CHO-SR cells and MSR-knockout macrophages (see Figs. 8 and 9), it is evident that GA-LDL is recognized and endocytosed by macrophages mainly through MSR, and then subjected to lysosomal degradation, followed by foam cell formation.

It is generally accepted that the negative charge of ligand is necessary, but not essential for ligand recognition by MSR (*2*). For instance, the maleylation or succinylation of

BSA resulted in an increase in the negative net charge and it became an effective ligand for MSR, whereas acetylation or reductive methylation of BSA increased the negative net charge to a similar extent, but failed to generate the ligand activity toward MSR (*57*). In this connection, modification of LDL with CML, one of the AGE-structures, led to an increase in the negative charge to an extent similar to acetyl-LDL, but it remained inert as a ligand for MSR (data not shown). Therefore, the structure(s) of GA-LDL responsible for recognition by MSR could be explained by a regional cluster(s) of negative charge on a GA-LDL particle, or by a specific structure(s) of GA-LDL. Westwood *et al.* recently made the interesting observation that the binding of methylglyoxal-modified BSA to THP-1 cells was competed for by an imidazolone derivative, one of the AGE-structures, indicating the importance of a specific AGE-structure as a ligand domain for AGE-receptors (*58*).

In the Maillard reaction, modified proteins up to Amadori products are usually called glycated proteins, whereas those after Amadori products (post-Amadori phase) are called AGE-modified proteins. The plasma level of glycated LDL was reported to be 2-fold higher in diabetic patients than in normal subjects (*59*). Witztum *et al.* prepared glycated LDL by incubating LDL *in vitro* with 80 mM glucose for 7 days in the presence of 12.5 mg/ml of cyanoborohydride (*6*). Their glycated LDL can be regarded as a product in the early phase, since a Schiff base was reduced to hexitol-lysine by cyanoborohydride and the rate of lysine modification determined by the TNBS method was fairly low (6-15%) (*6*). The endocytic degradation of this glycated LDL preparation by human fibroblasts was less than 10% of that of unmodified LDL, suggesting that the glycation of 6-15% of the total lysine residues of LDL readily leads to a significant loss of its ligand activity toward the LDL receptor (*6, 60*). The plasma clearance of their glycated LDL in guinea pigs was significantly retarded (2-3-fold) compared with that of unmodified LDL. These *in vitro* and *in vivo* experiments led to the general notion that the glycation of plasma LDL results in a decrease in its metabolic rate due largely to a significant loss of its ligand activity toward the LDL receptor system, with which the plasma LDL-cholesterol level becomes higher, thus facilitating the atherogenic process in diabetic vascular complications. Alternatively, it is possible that LDL modified by AGE, if it occurs *in vivo* in arterial walls, readily undergoes receptor-mediated endocytosis by monocyte-derived macrophages, which leads them to foam cells. This notion is supported by the present finding that GA-LDL, exhibiting high physicochemical and immunochemical similarity to AGE-proteins, is effectively endocytosed by mouse peritoneal macrophages, followed by foam cell formation. Thus, if glycated LDL is further modified *in vivo* to AGE-modified LDL through a reaction with intermediates of the Maillard reaction, it could play a direct role as an atherogenic lipoprotein in foam cell formation in early atherosclerotic lesions.

The presence of AGE-modified LDL in human plasma was reported by two groups. Gas chromatography/mass spectroscopic analyses by Lyons *et al.* demonstrated LDL modified with CML in normal as well as diabetic human plasma (*61*). ELISA involving a polyclonal antiserum against AGE-RNase (*62, 63*) successfully demonstrated a positive reaction in the LDL fraction, and showed that the amounts of plasma AGE-modified LDL in patients with renal insufficiency and diabetic renal insufficiency are 9- and 24-fold increased, respectively, compared with that in normal subjects (*63*). Bucala *et al.* also reported that the plasma clearance of ^{125}I-AGE-modified LDL in a mouse with the human LDL receptor-transgene took much longer than that of ^{125}I-LDL, whereas no significant difference in their plasma clearance was observed in wild-type mice, suggesting AGE-modified LDL is cleared from the circulation mainly *via* the LDL receptor-independent pathway (*63*). This was supported by the finding that the cellular binding of ^{125}I-LDL to human fibroblasts was not inhibited by their AGE-modified LDL preparation (*63*), again suggesting a significant reduction in the ligand activity toward the LDL receptor upon AGE-modification of LDL. However, these results are not consistent with those of our previous studies, in which ^{125}I-AGE-BSA was rapidly cleared from the circulation, with a half time of few minutes, in rats through the AGE-receptor system expressed by sinusoidal liver cells such as endothelial and Kupffer cells (*64, 65*). These inconsistent results could be explained by a difference in the extent of LDL-modification by AGE. Upon lysine modification by more than 60% by glycolaldehyde, LDL gained the new property of being recognized by macrophages through MSR (Fig. 8B). This finding, taken together with the previous results (*66, 67*), strongly suggests that lysine modification through glycation of apo B up to 25% is enough to reduce its ligand activity toward the LDL receptor (*66*), which includes 67 of the total 359 lysine residues of apo B (*68*), but further modification up to >60% is needed to generate the ligand activity toward MSR, suggesting that 25-60% lysine modification keeps LDL inert as to both the LDL receptor and MSR. A similar notion was presented by Haberland *et al.* (*67*); modification of <15% of the lysine residues of LDL with malondialdehyde had no effect on its ligand activity toward the LDL receptor, whereas further modification resulted in a threshold increase in recognition by the scavenger receptors with a concomitant loss of recognition by the LDL receptor. A similar threshold effect was observed for LDL modified with succinic anhydride and acetic anhydride (*67*). It is therefore likely that the extent of modification of the AGE-modified LDL prepared by Bucala *et al.* (*63*) was 25-60%, which is enough for the loss of its ligand activity toward the LDL receptor, but not enough to generate new ligand activity toward AGE-receptors.

Macrophage-derived foam cells are a prominent feature of the early stage of atherosclerotic lesions (*1*). Two important steps in atherogenesis include oxidative modification of LDL by arterial cells such as endothelial cells, smooth muscle cells and macrophages, and subsequent endocytic uptake of the Ox-LDL by macrophages through scavenger receptors (*69*). There are three lines of evidence for LDL oxidation *in vivo*. Firstly, Ox-LDL was found in human and rabbit atherosclerotic lesions on immunohistochemistry (*3, 70, 71*). Secondly, LDL fraction gently extracted from human and rabbit atherosclerotic lesions showed similar physicochemical properties, such as a higher electrophoretic mobility, a higher lysophosphatidylcholine content and apo B fragmentation, to Ox-LDL prepared by Cu^{2+}-oxidation *in vitro* (*4*). Thirdly, such a LDL fraction extracted from these lesions was effectively endocytosed by macrophages and induced CE accumulation

(*4*). The situation of AGE-modified LDL seems to be similar to that of Ox-LDL. Firstly our present data demonstrate that GA-LDL, prepared by incubating LDL with glycolaldehyde *in vitro*, directly underwent endocytic degradation by macrophages, followed by foam cell formation, suggesting possible linking of AGE-modified LDL to the progression of atherosclerotic lesions. Secondly, immunohistochemical studies involving anti-AGE antibodies have demonstrated the presence of AGE-structures in human atherosclerotic lesions (*7, 72*). Based on these results, AGE-modified LDL can be regarded as one of the atherogenic lipoproteins *in vivo*. Further studies are needed to resolve this issue.

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