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Interaction of Malondialdehyde-Modified Bovine Serum Albumin and Mouse Peritoneal Macrophages

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Reaction of bovine serum albumin (BSA) with malondialdehyde (MDA), a product of lipid oxidation, resulted in the modification of amino residues of the protein to produce three kinds of adducts in the protein molecules, aminopropenal (**1**), *N*,*N*′-disubstituted 1-amino-3-iminopropene (**2**) and 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde (**3**). Modified BSA, in which 39 out of the total of 60 amino residues were modified, showed effective binding to thioglycollate-induced mouse peritoneal macrophages. MDA-modified BSA inhibited the binding of formaldehyde-modified BSA to the macrophages, indicating that MDA-modified BSA binds to the scavenger receptor for formaldehyde-modified BSA. However, the converse was not the case, suggesting that MDA-modified BSA binds to additional receptors to which formaldehyde-modified BSA does not. Reduction of the double bonds of **1** and **2**, and the aldehyde functions of **1** and **3** in MDA-modified BSA did not affect the binding of the protein. However, modification of the aldehyde function of **1** with glycine resulted in loss of the ligand activity of the protein. These results suggest that adducts **1**, **2** and **3** in the BSA molecule are not directly involved in the binding to the scavenger receptor of the macrophages, though adduct **1** may be located near the binding site or may play a role in maintaining the active conformation of the binding site.

Keywords——macrophage; scavenger receptor; malondialdehyde; lipid oxidation; modified protein

Polyunsaturated fatty acids are liable to undergo oxidative deterioration, called lipid oxidation.[1,2] In this process, they break down to lipid hydroperoxides and various secondary products such as aldehydes and ketones.[1-3] The reactive molecules thus generated in living tissues can react with adjacent proteins to produce oxidized lipid-modified proteins.[4-6] Malondialdehyde (MDA), a lipid oxidation product,[2] is reactive with proteins to form cross-links and fluorescent chromophores,[4-7] and has been regarded as a molecule which is responsible for protein modification by oxidized lipids.[4,5]

It is known that serum albumin and low density lipoprotein (LDL) modified by acylating agents[8-10] or aldehydes[10-14] are bound and taken up by macrophages. Fogelman *et al.*[13] demonstrated that MDA-modified LDL, a model for oxidatively damaged LDL, was taken up by human monocyte-macrophages. The recognition and endocytosis of MDA-modified LDL are mediated by the macrophage surface receptor termed scavenger receptor.[15] It was hypothesized that charge modification of certain critical lysine residues of LDL apoprotein results in the formation of the determinants for the macrophage recognition.[16,17] However, the structural requirements of the MDA adducts formed in the apoprotein for the macrophage recognition are not known.

We have previously shown that three kinds of MDA adducts are produced by the reaction of MDA and polylysine (Fig. 1).[18] Adduct **1** is an aminopropenal residue produced by 1 : 1 addition of MDA molecules to ε-amino residues by Schiff base formation. Adduct **2** is a conjugated Schiff base, *N*,*N*′-disubstituted 1-amino-3-iminopropene, formed by 1 : 2 addition of MDA to ε-amino residues, which serves as a cross-link between polypeptides.[19]

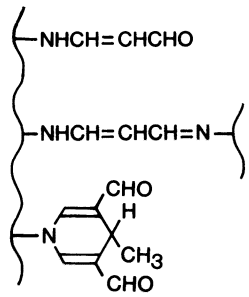

Fig. 1. Structures of Malondialdehyde Adducts Produced in the Reaction with Polylysine

Adduct **3** is a 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde residue produced by 3:1 addition of MDA to ε-amino residues, and is strongly fluorescent.[20,21]

In this report, we have investigated the binding of bovine serum albumin (BSA) modified by MDA to mouse peritoneal macrophages, and have studied the relationship between the structure of the MDA adducts and the binding activity of the modified protein.

Experimental

Materials——BSA was obtained from Sigma Chemical Company, St. Louis. MDA was prepared by acid hydrolysis of malonaldehyde bis(dimethylacetal)(Tokyo Kasei Kogyo, Tokyo) according to the method described previously.[22] Since MDA is unstable, it was prepared just before use. A portion of the acidic solution was diluted with 0.1 M sodium phosphate buffer (pH 6.5) to prepare 0.1 M MDA solution for use. Sodium borohydride was purchased from Kanto Chemical Company, Tokyo, and sodium cyanoborohydride from Aldrich Chemical Company, Milwaukee. Sodium [^{125}I]iodide (356 mCi/ml, carrier-free) was from New England Nuclear, Boston. Hanks' balanced salt solution and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) were from Nissui Pharmaceutical Company, Tokyo, and Dojindo Laboratories, Kumamoto, respectively. RPMI-1640 medium and fetal calf serum were obtained from GIBCO Laboratories, Chagrin Falls. All other chemicals were reagent-grade products of Wako Pure Chemical Industries, Osaka.

Preparation of MDA-Modified BSA——A solution of BSA (20 mg/ml) in 0.1 M sodium phosphate buffer (pH 6.5) was mixed with an equal volume of 20 or 50 mM MDA solution in the same buffer. The mixture was allowed to stand at 37 °C for 24 h, followed by dialysis against 0.1 M sodium phosphate buffer (pH 7.5) at 4 °C to remove free MDA, and the MDA-modified BSA solution was stored at −80 °C until use. Reduction of MDA-modified BSA was carried out by incubation either with 50 mM sodium borohydride at 25 °C for 4 h or with 50 mM sodium cyanoborohydride at 25 °C for 18 h in the dark. After the reduction, the reaction mixtures were extensively dialyzed against 0.1 M sodium phosphate (pH 7.5) at 4 °C, and stored at −80 °C. A portion of MDA-modified BSA was allowed to react with glycine by further incubation with 0.5 M glycine in 0.1 M sodium phosphate (pH 7.5) at 37 °C for 24 h. After the incubation, free glycine was removed by dialysis against the same buffer. Reduction of glycine-treated MDA-modified BSA was performed in the same fashion as that of MDA-modified BSA.

Analysis of MDA-Modified BSA——Protein concentration of MDA-modified BSA was determined by the method of Lowry *et al.*[23] using unmodified BSA as a standard. The number of free amino residues of the modified BSA was determined by the trinitrobenzenesulfonic acid method according to Habeeb.[24] Unmodified BSA (60 amino residues/molecule[25]) was used as a standard, and the number of modified amino residues was calculated as the difference in values obtained for the modified and unmodified BSA. The number of aminopropenal residues in MDA-modified BSA was estimated from the increase in ultraviolet (UV) absorption at 282 nm.[18] The extinction coefficient of the standard aminopropenal of α-acetyllysine methylester, log ε 4.53 (282 nm)[26] was used for calculation. The amount of the fluorescent chromophore, 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde, in the MDA-modified BSA was determined fluorometrically[18] using a relative molar intensity of the fluorophore against quinine sulfate, of 1.47 (excitation at 398 nm, emission at 460 nm).[21] The measurement of the fluorescence was done in 0.1 M sodium phosphate buffer (pH 7.5) containing 5% sodium dodecyl sulfate (SDS) since denaturation of MDA-modified BSA by SDS was necessary in order to obtain a proper fluorescence spectrum of the chromophore. The extent of intermolecular cross-linking of the modified BSA was estimated by SDS–polyacrylamide gel electrophoresis by the method of Laemmli[27] using a 4% stacking gel and a 7.5% separating gel.

Preparation of Formaldehyde-Modified BSA (f-BSA)——f-BSA was prepared according to Horiuchi *et al.*[14] in sodium carbonate buffer (pH 10.0) at 37 °C for 1 h. After dialysis against 0.1 M NaCl and centrifugation, the clear supernatant was stored at −80 °C until use. Protein concentration and the number of modified amino residues were determined as described above. In our preparation, 32 amino residues per BSA molecule (53.3% of the total amino

residues) were modified by formaldehyde, which is comparable to the reported value (46.2%).[14] Reduction of f-BSA was performed as described for MDA-modified BSA.

Radioiodination——Radiolabeling of the modified BSA was performed using $Na^{125}I$ by the chloramine-T method.[28] The labeled protein was diluted with the unlabeled protein to give a specific activity of $5—10 \times 10^4$ cpm/μg prior to use.

Macrophages——Macrophages were obtained from the peritoneal cavity of 7- to 10-week-old ddY mice 4 d after an intraperitoneal injection of 2—3 ml of 3% thioglycollate medium (Difco Laboratories, Detroit). Peritoneal exudate cells obtained were washed twice with Hanks' balanced salt solution by centrifugation at 4 °C. The cells were then resuspended in RPMI 1640 medium supplemented with 20 mM Hepes (pH 7.2) 50 units/ml penicillin and 50 μg/ml streptomycin (RPMI-Hepes medium) at 1×10^6 cells/ml, and aliquots of 3 ml of the cell suspension were layered in a 60 mm plastic Petri dish (Corning Glass Works, Corning). After incubation at 37 °C for 1 h, nonadherent cells were removed by washing three times with 10 mM phosphate-buffered saline (pH 7.2) and the adherent cells were further incubated at 37 °C for 2 h in RPMI-Hepes medium supplemented with 5% heat-inactivated fetal calf serum. To measure the number of adherent cells, the monolayer cells were scraped off, suspended and counted. Usually $1.7—2.0 \times 10^6$ cells/dish was obtained.

Binding Assay——Binding of the modified BSA to the macrophage monolayers was performed at 0—4 °C to prevent subsequent endocytosis of the bound ligands. Macrophage monolayers that had been incubated at 37 °C overnight were chilled on ice for 30 min before use. The culture medium was replaced by 1 ml of precooled RPMI-Hepes medium containing 1% BSA and ^{125}I-labeled modified BSA. To estimate nonspecific binding, some dishes received an excess amount (2 mg/ml) of unlabeled modified BSA. For the competitive binding experiment, various concentrations of unlabeled modified BSA competitors were included in the replacing medium. After the incubation on ice for 2 h with occasional shaking, the radioactive medium was removed and the macrophage monolayers were washed 4 times with precooled 10 mM phosphate-buffered saline (pH 7.2). The washed monolayers were then solubilized in 2 ml of 1 N NaOH, the radioactive solutions were transferred to γ-ray counting vials, and the radioactivity was counted. Adsorption of the radioactivity on the Petri dish was similarly measured using the dish without macrophages, and this value was subtracted from the data as a blank. The data shown are the mean values of duplicate determinations.

Results

BSA was modified by 10 and 25 mM MDA at 37 °C for 24 h, and the resultant MDA-modified BSA, MDA-BSA [I] and MDA-BSA [II], respectively, were analyzed to determine the degree of modification (Table I). In the modification by 10 mM MDA (MDA-BSA [I]), 26 amino residues out of the total of 60 amino residues (59 lysines and 1 amino terminal[25]) of the protein were modified by MDA. The number of aminopropenal residues (adduct **1**) was about 11, while that of fluorescent 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde residues (adduct **3**) was 0.3. The remaining 14 amino residues modified may be involved in intra- or intermolecular cross-links of *N*,*N*′-disubstituted 1-amino-3-iminopropene (adduct **2**). SDS–polyacrylamide gel electrophoresis of MDA-BSA [I] indicated that intermolecularly cross-linked BSA oligomers which appeared above the monomer band on the gel were produced. The number of modified amino residues increased to 39 mol/mol BSA when BSA was modified by 25 mM MDA (MDA-BSA [II]). The number of fluorophores also increased while that of aminopropenal residues unchanged. Consistent with the increase in the number of

TABLE I. Molecular Features of MDA-Modified BSA

Analysis	MDA-BSA [I][a]	MDA-BSA [II][b]
Amino residues modified[c] (mol/mol BSA)	25.8	39.1
Aminopropenal residues (mol/mol BSA)	11.3	11.2
1,4-Dihydropyridine-3,5-dicarbaldehyde residues (mol/mol BSA)	0.3	0.8
Intermolecular cross-links[d]	+	++

a) BSA modified by 10 mM MDA at pH 6.5 and 37 °C for 24 h. *b*) BSA modified by 25 mM MDA at pH 6.5 and 37 °C for 24 h. *c*) Determined by the trinitrobenzenesulfonic acid method[24] using unmodified BSA (60 amino residues/molecule) as a standard. *d*) Determined by SDS-polyacrylamide gel electrophoresis. +, BSA oligomer bands were observed above the monomer band on the gel. ++, in addition to the oligomer bands, BSA polymers were observed at the top of the gel.

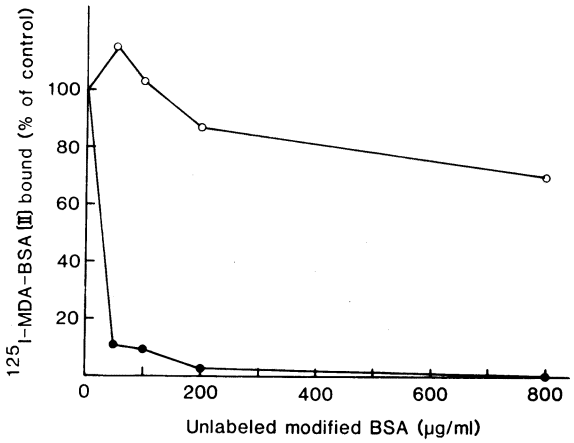

Fig. 2. Ability of MDA-BSA [I] and [II] to Inhibit the Binding of ^{125}I-MDA-BSA [II] to Mouse Peritoneal Macrophages

The binding of ^{125}I-MDA-BSA [II] to monolayers of thioglycollate-induced mouse peritoneal macrophages was measured at 0—4 C for 2 h in the presence of the indicated concentrations of unlabeled MDA-BSA [I] (○) and [II] (●). The concentration of ^{125}I-MDA-BSA [II] used was 10 μg/ml.

modified amino residues, a small amount of BSA polymers was detected at the top of the gel in SDS–polyacrylamide gel electrophoresis.

Binding activity of MDA-BSA [II] to mouse macrophages was examined using monolayers of thioglycollate-induced mouse peritoneal macrophages at 0—4 °C, at which temperature endocytosis does not take place.[11] ^{125}I-labeled MDA-BSA [II] (^{125}I-MDA-BSA [II]) bound to these cells depending upon its concentration. At the ^{125}I-MDA-BSA [II] concentration of 10 μg/ml, the specific binding to the cells which was inhibitable by an excess amount of unlabeled MDA-BSA [II] (2 mg/ml) was 45—75 ng/10^6 cells. The binding activity of the modified BSA depended on the degree of modification. As shown in Fig. 2, the binding of ^{125}I-MDA-BSA [II] was effectively inhibited by unlabeled MDA-BSA [II], whereas the binding was poorly inhibited by the less modified BSA (MDA-BSA [I]). The striking difference in inhibitory activity between these two preparations may indicate that there is a threshold value of the number of amino groups modified of between 26 and 39 per BSA for the macrophage recognition of MDA-modified BSA (see Table I).

It is known that macrophages have the scavenger receptor for f-BSA.[12,29] To examine the specificity of MDA-BSA [II] in the binding to the mouse peritoneal macrophages, competitive binding experiments of MDA-BSA [II] and f-BSA were carried out. Unlabeled MDA-BSA [II] inhibited the binding of ^{125}I-labeled f-BSA (^{125}I-f-BSA) to the macrophage monolayers as effectively as unlabeled f-BSA (Fig. 3A). However, unlabeled f-BSA was ineffective in inhibiting the binding of ^{125}I-MDA-BSA [II] to the macrophages while unlabeled MDA-BSA [II] was effective (Fig. 3B). The competitive binding activity of f-BSA reduced with borohydride or cyanoborohydride was then examined. Borohydride reduces double bonds of Schiff bases and aldehyde functions, while cyanoborohydride reduces only Schiff bases.[30,31] The reduced f-BSA, which retained the activity to compete with f-BSA (Fig. 3A),[14] was also ineffective in competing with MDA-BSA [II] (Fig. 3B). These results suggest that MDA-BSA [II] binds to the scavenger receptor for f-BSA, and to additional receptor sites to which f-BSA do not bind.

To examine the relationship between the structure of the MDA adducts (adducts **1**, **2** and **3**) and the receptor binding activity of MDA-BSA [II], MDA-BSA [II] was treated with sodium borohydride or sodium cyanoborohydride, and then tested for ligand activity in competitive binding experiments with ^{125}I-MDA-BSA [II]. Reduction of MDA-BSA [II] with borohydride resulted in the loss of its fluorescence, due to reduction of one of the two aldehyde moieties of adduct **3**,[32] while reduction with cyanoborohydride did not affect the fluorescence. The ligand activity of MDA-BSA [II] was little affected by either reduction (Fig. 4). This indicates that neither the double bonds of **1** and **2** nor the aldehyde moieties of **1** and **3**

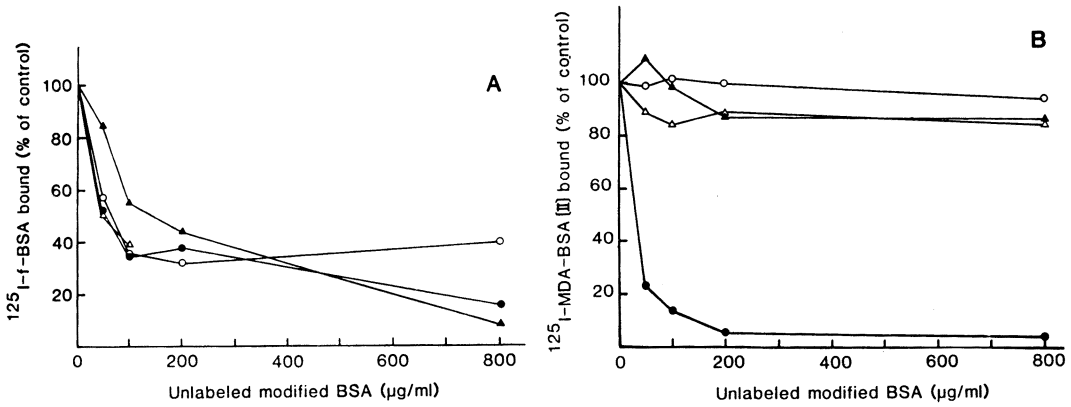

Fig. 3. Effects of MDA-BSA [II], f-BSA, and Reduced f-BSA on the Binding of ^{125}I-f-BSA (A) and ^{125}I-MDA-BSA [II] (B) to Mouse Peritoneal Macrophages

The binding experiments of ^{125}I-f-BSA (10 μg/ml) (A) and ^{125}I-MDA-BSA (10 μg/ml) (B) were performed in the presence of unlabeled modified BSA as described in the legend to Fig. 2. The amount of ^{125}I-f-BSA at 10 μg/ml bound in the absence of unlabeled competitors was 10 ng/10^6 cells. ●, MDA-BSA [II]; ○, f-BSA; ▲, f-BSA reduced with $NaBH_4$; △, f-BSA reduced with $NaBH_3CN$.

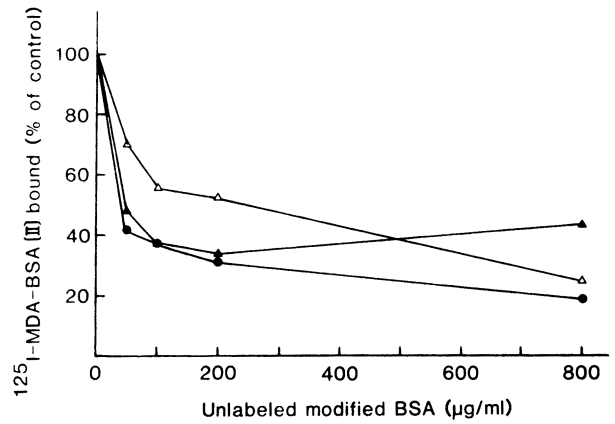

Fig. 4. Effect of Reduction of MDA-BSA [II] on Its Binding Activity to Mouse Peritoneal Macrophages

MDA-BSA [II] was reduced with $NaBH_4$ or $NaBH_3CN$, and the ability to inhibit the binding of ^{125}I-MDA-BSA [II] (10 μg) to mouse peritoneal macrophages was measured as described in the legend to Fig. 2. ●, MDA-BSA [II]; ▲, MDA-BSA [II] reduced with $NaBH_4$; △, MDA-BSA [II] reduced with $NaBH_3CN$.

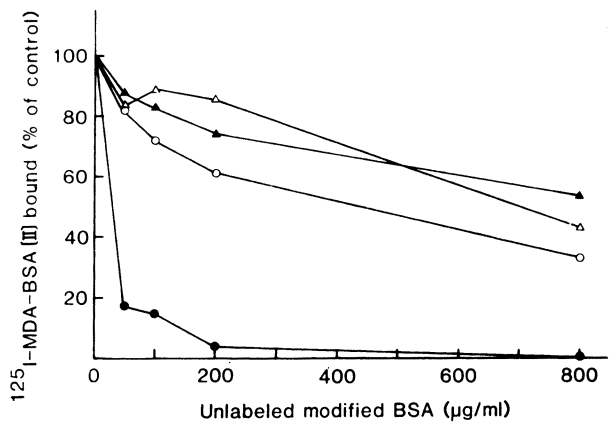

Fig. 5. Effect of Modification of MDA-BSA [II] by Glycine on Its Binding Activity to Mouse Peritoneal Macrophages

MDA-BSA [II] was modified by glycine or by glycine followed by reduction as described in Experimental, and the ability to inhibit the binding of ^{125}I-MDA-BSA [II] (10 μg/ml) to mouse peritoneal macrophages was measured as described in the legend to Fig. 2. ●, MDA-BSA [II]; ○, MDA-BSA [II] modified by glycine; ▲, MDA-BSA [II] modified by glycine followed by reduction with $NaBH_4$; △, MDA-BSA [II] modified by glycine followed by reduction with $NaBH_3CN$.

are essential for the ligand activity. Furthermore, this suggests that neutralization of one of the positive charges of two lysyl residues by the formation of a conjugated Schiff base ($2\text{–}NH_3^+ + OHCCH_2CHO \rightarrow \text{–}NH_2{}^+CH=CHCH=N\text{–}$) is not essential for the ligand ac-

tivity, since subsequent reduction restores the positive charge ($-NH_2^+CH=CHCH=N-\rightarrow -NH_2^+CH_2CH_2CH_2NH_2^+-$).

It is known that aminopropenal residues can react with free amino groups to form conjugated Schiff bases.[18] The effect of modification of adduct **1** was examined by the incubation of MDA-BSA [II] with glycine ($-NH_2^+CH=CHCHO+H_3^+NCH_2COO^-\rightarrow -NH_2^+CH=CHCH=NCH_2COO^-$). On modification by glycine, the ligand activity of MDA-BSA [II] was markedly diminished (Fig. 5). The glycine-treated MDA-BSA [II] was then reduced with borohydride or cyanoborohydride to cancel out the negative charge due to the carboxyl group of glycine. The reduction did not affect the diminished ligand activity (Fig. 5). Thus, the loss of the ligand activity of MDA-BSA [II] on treatment with glycine is not likely to be due to the alteration of charge of adduct **1**, but may be due to a secondary effect on the protein structure.

Discussion

MDA is one of the agents which may modify proteins and LDL apoprotein during lipid oxidation *in vivo*.[4,5,13] In the present study, *in vitro* modification of BSA with MDA resulted in the conversion of the protein to the species recognized by thioglycollate-induced mouse peritoneal macrophages. An effective binding was achieved by the modification of 39 amino residues/BSA molecule while the modification of 26 amino residues/BSA molecule was less effective. There may be a threshold for the macrophage recognition between the two values. Haberland *et al.*[16] reported that MDA-modified LDL was recognized by human monocyte-macrophages when the modification exceeded a threshold of 30 mol MDA/mol LDL.

Eskild and Berg reported that endocytosis of f-BSA by rat nonparenchymal liver cells was inhibited by MDA-modified BSA,[33] which indicated that MDA-modified BSA bound to the scavenger receptor for f-BSA. In agreement with their observation, binding of f-BSA to the mouse peritoneal macrophages was inhibited by MDA-BSA [II]. However, f-BSA was unable to inhibit the binding of MDA-BSA [II]. A likely explanation for the result is that MDA-BSA [II] binds not only to the scavenger receptor for f-BSA but also to other receptor sites. It is unlikely that MDA-BSA [II] nonspecifically binds to the macrophages by covalent attachment, which would not be inhibited by f-BSA, due to Schiff base formation between the aldehyde moieties of the modified protein and amino groups of the cell surface components, because reduction of MDA-BSA [II] with borohydride did not affect its ligand activity.

So far there has been little evidence demonstrating the recognition of aldehyde adducts in the aldehyde-modified proteins by the macrohage scavenger receptor. Inasmuch as the structure of MDA adducts formed by the reaction of MDA and amino compounds has been clarified,[7,18-21] it was possible to examine the relationship between the structure of the adducts and the ligand activity of the MDA-modified BSA. The structural changes of the three kinds of adducts (**1**, **2** and **3**) upon reduction did not affect the ligand activity of MDA-BSA [II]. Therefore, these adducts may not be directly involved in the interaction with the macrophage receptor. Haberland and Fogelman[34] reported that maleylated BSA, which binds to the receptor for MDA-LDL on human monocyte-macrophages, retained the ligand activity after demaleylation, suggesting that the receptor does not recognize maleyl residues but does recognize conformationally altered BSA polypeptides. It is conceivable that the modification of the threshold number of lysyl residues of BSA by MDA causes a conformational change of the protein which results in the formation of the binding sites for the macrophage receptor. The difference in the number of lysyl residues modified between MDA-BSA [II] and [I], an active and a less active derivative, respectively, appears to be due to the difference in the content of adduct **2** (inter- or intramolecular cross-links) since the content of adduct **1** was the same. Therefore, formation of adduct **2** may be the effective modification of

the protein causing such conformational change. The fact that elongation of aminopropenal residues with glycine diminished the ligand activity suggests that the residue is located near the binding site, and the elongation of the residue interferes with the interaction of the binding site and the receptor, or alternatively, the residue is necessary to maintain the binding site in an active form, and the elongation of the residue may lead to the destruction of the active conformation of the binding site.

It has been considered that negative charges of ligands are essential for the interaction with the scavenger receptor of macrophages[8-10] since the effective modification of proteins and LDL for generation of the ligand activity involves reaction with lysyl residues,[8,9,13,16,17] and some polyanionic macromolecules such as polyinosinic acid and dextran sulfate are effective competitors for the ligand binding.[8,9] However, the loss of the positive charges of lysyl residues due to the conjugated Schiff base formation with MDA may not be essential for the interaction of MDA-BSA [II] and the scavenger receptor because i) reduction of the double bonds of the conjugated Schiff bases restored the positive charges at the lysyl residues but the ligand activity of MDA-BSA [II] did not diminish, and ii) introduction of negatively charged carboxyl groups at aminopropenal residues by modification with glycine did not increase but decreased the ligand activity of MDA-BSA [II].

Further experimentation is necessary to clarify the structural requirements of the adducts for the formation of the binding sites.

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