Legends for Figures:

Figure 1. The scheme for the reaction of 2-iminothiolane with primary amines followed by S-nitrosylation

Figure 2. Production of ROS in C26 cells after NO-HSA treatment

C26 cells were pre-treated with H_2DCFDA for uptake into C26 cells and hydrolysis by cellular esterase, followed by addition of either PBS (*open circles*), 50 μM HSA (*open squears*) or 50 μM NO-HSA (*closed circles*). Excitation of the probes was done at 485 nm and emission was measured at 535 nm. Change in fluorescence was calculated by subtracting the fluorescence at 0 h from that at the indicated times. The fluorescence intensities of the PBS, 50 μM HSA and 50 μM NO-HSA groups at 0 h were 201.3, 166.1 and 181.3, respectively. Results are the mean ± S.D. of three separate experiments.

Figure 3. Induction of apoptosis of C26 cells after NO-HSA treatment

(A) Alteration in the mitochondrial membrane potential after NO-HSA treatment

C26 cells were cultured with PBS, 100 μM HSA or various concentrations of NO-HSA for 24 h, followed by addition of rhodamine 123. The amounts of cell-associated rhodamine 123 were determined as described in Materials and Methods. Results are the mean ± S.D. of three separate experiments.

(B) Activation of caspase-3 after NO-HSA treatment

C26 cells were incubated with PBS, 100 μM HSA or various concentrations of NO-HSA for 24 h. Caspase-3 activity was estimated by monitoring *p*-nitroaniline (absorbance at 405 nm) released from the substrate upon cleavage by caspase-3. Change

in absorbance was calculated by subtracting absorbance after incubation with caspase inhibitor (Z-VAD-FMK), from absorbance after incubation without caspase inhibitor. The absorbances among PBS-, HSA- and NO-HSA-treated cells incubated with Z-VAD-FMK were almost identical (0.170 ± 0.17). Results are the means ± S.D. of three separate experiments.

(C) DNA fragmentation after NO-HSA treatment

C26 cells were incubated with PBS, 100 μM HSA or various concentrations of NO-HSA for 24 h. DNA fragmentation was detected as described in the Materials and Methods.

Figure 4. Effect of NO-HSA on C26 cell viability

(A) C26 cells were treated for 48 h with various concentrations of HSA (*open squares*) or NO-HSA (*closed circles*). (B) C26 cells were incubated for the indicated times with 100 μM HSA (*open squares*) or 100 μM NO-HSA (*closed circles*). Cell viability was determined as described in the Methods. Results are the mean ± S.D. of three separate experiments.

Figure 5. Effect of NO-HSA on tumor growth in C26 tumor-bearing mice

C26 tumor-bearing mice were given daily intravenous injections of saline (5 ml/kg), HSA (10 μmol/5 ml/kg) or NO-HSA (10 μmol/5 ml/kg) for ten days from day 3 to day12 after inoculation with tumor cells. Tumor size was measured and tumor volume was calculated according to the formula: $V = 0.4a^2b$, where a is the smallest, and b is the largest, superficial diameter. Results are means ± S.D; n = 10 animals per

experimental group. *Statistically significant reduction compared with saline ($P<0.01$) or HSA ($P<0.05$) at the corresponding time. **Statistically significant reduction compared with saline ($P<0.01$) or HSA ($P<0.01$) at the corresponding time.

Figure 6. Immunohistochemical staining of tumor tissues of C26 tumor-bearing mice intravenously injected with NO-HSA using the TUNEL method.

C26 tumor-bearing mice were given daily intravenous injections of saline (5 ml/kg) (A), HSA (10 μmol/5 ml/kg) (B) or NO-HSA (10 μmol/5 ml/kg) (C) for five days from day 3 to day 7 after inoculation with tumor cells. TUNEL-staining, performed as described in the Materials and Methods, shows apoptotic cells in the tumor tissue of mice treated with NO-HSA.

Table

Table 1. Serum biochemical parameters of C26 tumor-bearing mice treated with saline, HSA or NO-HSA

C26 tumor-bearing mice were intravenously injected five times per day with saline (5 ml/kg), HSA (10 μmol/5 ml/kg) or NO-HSA (10 μmol/5 ml/kg) on days from 3 to 7 after inoculation with tumor cells. Blood samples were collected from the abdominal vena cava under anesthesia with diethylether approximately 2 h after the last treatment injection and mice were sacrificed. Serum biochemical parameters were measured using routine clinical laboratory techniques.

	Saline	HSA	NO-HSA
Total protein (g/dl)	5.23 ± 0.21	5.33 ± 0.06	5.55 ± 0.24
Cr (mg/dl)	0.13 ± 0.04	0.14 ± 0.01	0.16 ± 0.01
BUN (mg/dl)	15.25 ± 0.96	15.33 ± 1.53	13.75 ± 1.26
AST (U/l)	143.5 ± 62.9	91.7 ± 39.4	116.3 ± 69.1
ALT (U/l)	169.8 ± 111.6	110.0 ± 81.2	161.3 ± 126.2
ALP (U/l)	385.3 ± 18.3	345.3 ± 4.7[a]	300.5 ± 23.0[b,c]

a; $p<0.05$, Saline vs HSA, b; $p<0.01$, Saline vs NO-HSA, c; $p<0.05$, HSA vs NO-HSA

Figure 1

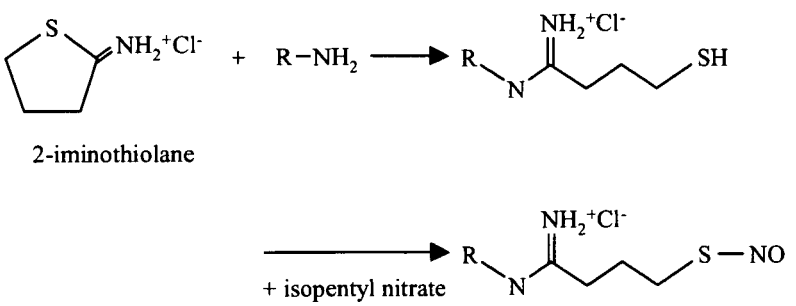

S
NH2+Cl-
+
R–NH2
R
N
NH2+Cl-
SH
2-iminothiolane
+ isopentyl nitrate
S—NO

Figure 2

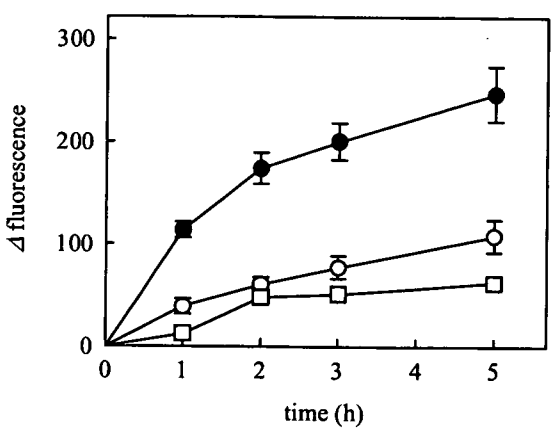


Figure 3 (A)

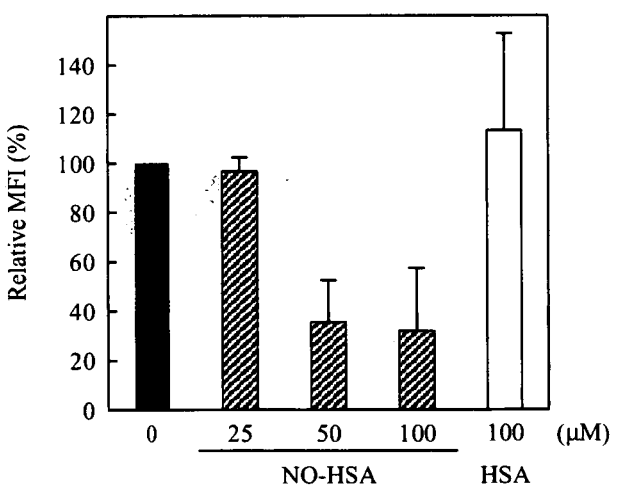

Relative MFI (%)
140
120
100
80
60
40
20
0
0
25
50
100
100
(μM)
NO-HSA
HSA

Figure 3 (B)

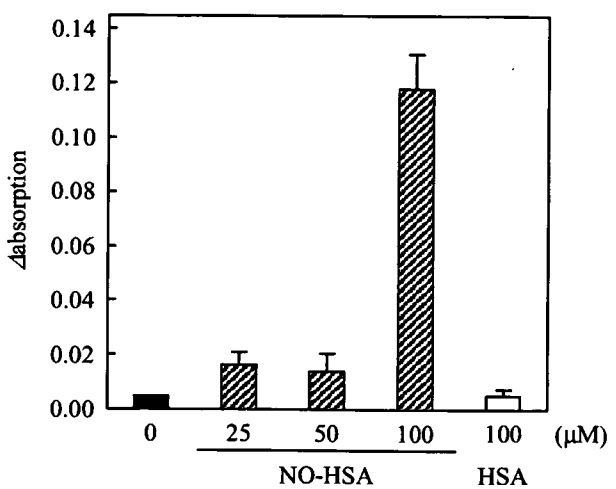

0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Δabsorption
0
25
50
100
100
(μM)
NO-HSA
HSA

Figure 3 (C)

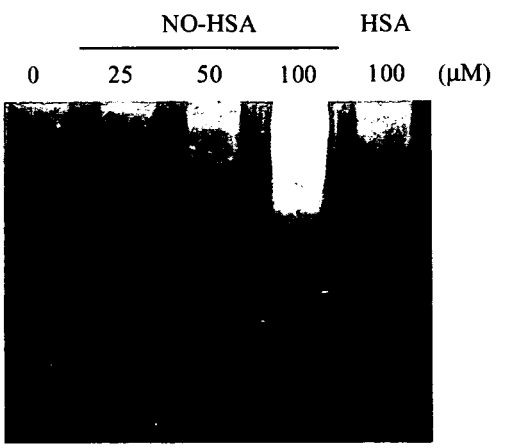


Figure 4

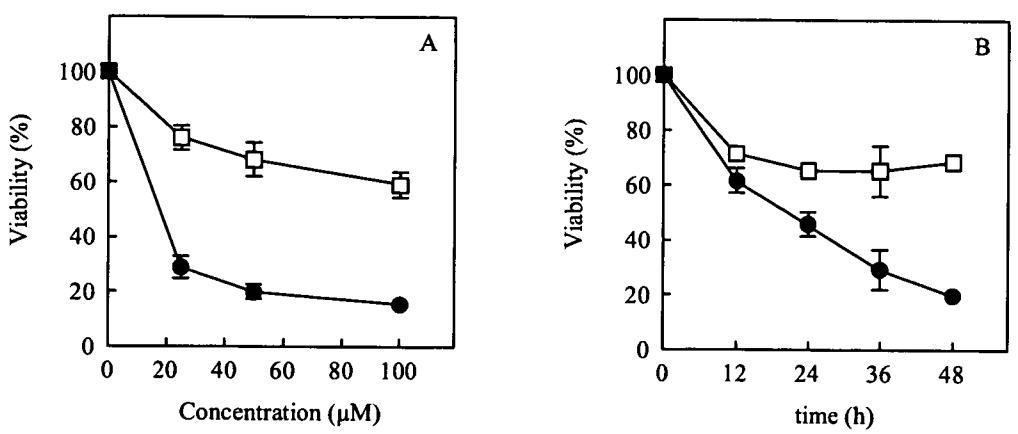

A
Viability (%)
100
80
60
40
20
0
0
20
40
60
80
100
Concentration (μM)
B
Viability (%)
100
80
60
40
20
0
0
12
24
36
48
time (h)

Figure 5

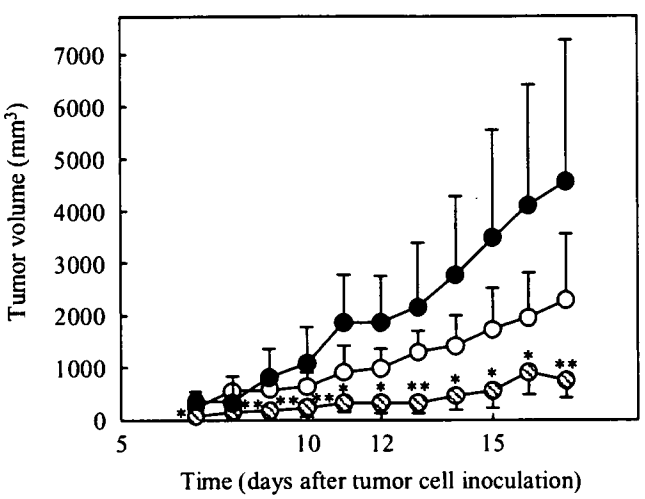
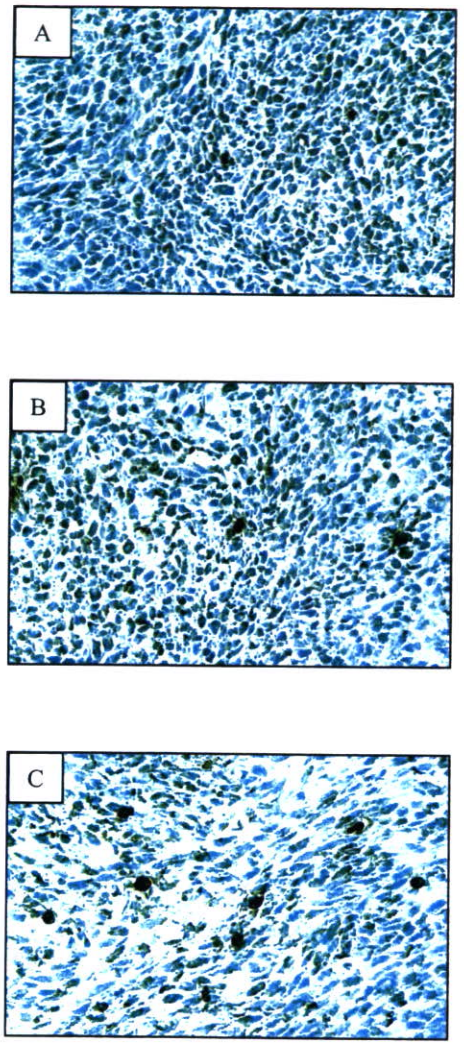

Tumor volume (mm3)
7000
6000
5000
4000
3000
2000
1000
0
5
10
12
15
Time (days after tumor cell inoculation)

Figure 6



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The ligand activity of AGE-proteins to scavenger receptors is dependent on their rate of modification by AGEs

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Abstract

The cellular interaction of proteins modified with advanced glycation end-products (AGEs) is believed to induce several different biological responses, which are involved in the development of diabetic vascular complications. We report here that the ratio of protein glycation is implicated in its ligand activity to scavenger receptors. Although highly-modified AGE-bovine serum albumin (high-AGE-BSA) was significantly recognized by human monocyte-derived macrophages and Chinese hamster ovary cells which overexpress such scavenger receptors as CD36, SR-BI (scavenger receptor class B type-I), and LOX-1 (Lectin-like Ox-LDL receptor-1), the mildly-modified-AGE-BSA (mild-AGE-BSA) did not show any ligand activity to these cells. Furthermore, when ^{111}In-labeled high- or mild-AGE-BSA were injected into the tail vein of mice, the high-AGE-BSA was rapidly cleared from the circulation whereas the clearance rate of the mild-AGE-BSA was very slow, similar to the native BSA. These results demonstrate the first evidence that the ligand activity of the AGE-proteins to the scavenger receptors and its pharmacokinetic properties depend on their rate of modification by AGEs, and we should carefully prepare the AGE-proteins *in vitro* to clarify the physiological significance of the interaction between the AGE-receptors and AGE-proteins.

Keywords: Advanced glycation end products (AGEs); N^{ε}-(carboxymethyl)lysine (CML); Scavenger receptor; Macrophage; Atherosclerosis; Diabetes

1. Introduction

The interaction between proteins modified with advanced glycation end-products (AGEs) and the scavenger receptor(s) of macrophages and smooth muscle cells is known to induce the production of several cytokines such as plasminogen activator and transforming growth factor-beta [1]. Furthermore, glycolaldehyde-derived AGE-low-density lipoprotein (AGE-LDL) induces foam cell formation from macrophages [2]. These findings suggest the role of the AGE-modified proteins and lipoproteins in the pathogenesis of atherosclerosis. However, AGE-proteins are prepared in each research group independently with different protocols, and all researchers, including our group, use excessively high concentrations of glucose and aldehydes. For instance, we prepared highly-modified AGE-bovine serum albumin (high-AGE-BSA) by incubating BSA with 1600 mM glucose for 40 weeks, or 33 mM glycolaldehyde for 7 days [3], and demonstrated that high-AGE-BSA is recognized by SR-A (class A scavenger receptor types I and II) [4], CD36 [5], SR-BI (scavenger receptor class B type-I) [6], and LOX-1 (Lectin-like Ox-LDL receptor-1) [7]. Schmidt et al. demonstrated that AGE-BSA, prepared by incubating BSA with 250 mM glucose-6-phosphate for 4 weeks, is recognized by RAGE (receptor for AGE) [8] and the cellular interactions of AGEs with RAGE are known to induce several cellular phenomena including the expression of vascular cell adhesion molecule-1 (VCAM-1) in

Abbreviations: AGE(s), advanced glycation end products; BSA, Bovine serum albumin; High-AGE-BSA, highly modified AGE-BSA; Mild-AGE-BSA, Mildly modified AGE-BSA; GA-AGE-BSA, Glycolaldehyde-derived AGE-BSA; MALDI-TOFMS, Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry; CML, N^{ε}-(carboxymethyl)lysine; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline

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endothelial cells [9], and cytokines in monocytes [10]. Vlassara and her colleagues prepared AGE-BSA by incubating BSA with 50 mM glucose-6-phosphate for 6 weeks [11] and demonstrated that AGE-receptor 1 (AGER1), a 50-kDa type A integral membrane protein with a short internal domain, suppresses cell oxidant stress and activation signaling via the EGF receptor [12]. Furthermore, Takeuchi et al. also demonstrated that AGE-BSA prepared using an unphysiologically high concentration of glyceraldehydes results in toxicity to the vascular wall cells and cortical neurons [13]. Taken together, those reports demonstrate that the AGE-proteins were prepared with unphysiologically high concentrations of aldehydes and then were employed for the cellular experiments. However, little is known about the correlation of the ligand activity of AGE-proteins and its rate of modification. In the present study we prepared mildly modified AGE-BSA (mild-AGE-BSA) by incubating BSA with 50 mM glucose for 24 weeks, and then compared its ligand activity with high-AGE-BSA against the scavenger receptors.

2. Materials and methods

2.1. Chemicals

D-glucose was purchased from Sigma-Aldrich Japan (Tokyo). Fatty acid-free bovine serum albumin (BSA) was purchased from Wako (Osaka, Japan). Lipopolysaccharide (LPS) from *Escherichia coli* was purchased from Sigma-Aldrich Japan (Tokyo). Na ^{125}I was obtained from Amersham Biosciences (Arlington Heights, IL). Tissue culture medium was from Gibco BRL (NY, USA). All other chemicals were of the best grade available from commercial sources.

2.2. Preparation of AGE-modified BSA

High-AGE-BSA was prepared as described previously [1,3]. Briefly, 0.2 g/ml of fatty acid-free BSA was dissolved in 0.5 M sodium phosphate buffer (pH 7.4) with 1.6 M of D-glucose, sterilized by ultrafiltration and incubated at 37 °C for 40 weeks, followed by dialysis against PBS. Mild-AGE-BSA was prepared by incubating 0.05 g/ml of fatty acid-free BSA with 50 mM of glucose in a 0.05 M sodium phosphate buffer (pH 7.4) at 37 °C for 24 weeks, followed by dialysis against PBS. The glycolaldehyde-derived AGE-BSA (GA-AGE-BSA) was prepared by incubating 2 mg/ml of fatty acid-free BSA with 30 mM of GA in a 0.05 M sodium phosphate buffer (pH 7.4) at 37 °C for up to 7 days, and aliquots of the samples were collected at 3 h, 6 h, 12 h, 24 h, 48 h, 96 h, and 168 h, followed by dialysis against PBS. The extent of lysine modification of the proteins was determined by reaction with 2, 4, 6-trinitrobenzenesulphonic acid as described previously [3].

2.3. Characterization of aldehyde-modified proteins

The lysine and N^{ε}-(carboxymethyl)lysine (CML) contents in the AGE-BSA and human lens samples were quantified by high performance liquid chromatography (HPLC) after acid hydrolysis with 6 N HCl for 24 h at 110 °C, as described previously [14]. The molecular mass of the AGE-BSA was measured as described previously [15]. Briefly, 1 μl of the sample protein solution (10 pmol/μl) in 0.1% aqueous trifluoroacetic acid (TFA) water was applied on a sample spot in a steel plate slide and dried in a stream of warm air. The matrix solution which contained 10 mg/ml 3,5-dimethoxy-4-hydroxycinnanic acid (sinapinic acid) (Sigma-Aldrich Japan) in 50% vol/vol ethanol in 0.1% vol/vol TFA was applied on a dried sample spot then dried in a stream of warm air again. The molecular mass of the sample was analyzed by Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOFMS) (Shimadzu/Kratos Kompact MALDI III mass spectrometer, Kyoto, Japan), operating in the positive high energy linear mode. An agarose gel electrophoresis was performed using the Universal Gel/8 electrophoresis kit (Ciba-Corning, Tokyo), followed by staining with Coomassie brilliant blue. Their electrophoretic mobilities relative to native BSA were expressed as the relative electrophoretic mobility.

2.4. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as described previously [16]. Briefly, each well of a 96-well microtiter plate was coated with 100 μl of the sample to be tested in PBS, blocked with 0.5% gelatin, and washed three times with PBS containing 0.05% Tween 20 (washing buffer). The wells were incubated with 0.1 ml of monoclonal anti-AGE antibody (6D12: 0.5 μg/ml) dissolved in washing buffer for 1 h. The wells were then washed with washing buffer three times and reacted with HRP-conjugated anti-mouse IgG antibody, followed by reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated by the addition of 0.1 ml of 1 M sulfuric acid, and the absorbance at 492 nm was read by a micro-ELISA plate reader. We recently reported that 6D12 significantly recognizes CML and N^{ε}-(carboxyethyl)lysine [16].

2.5. Cellular assays

The modified BSA preparations were radiolabeled with ^{125}I using Iodo-Gen (Pierce, Rockford, IL) and dialyzed against PBS. The CHO cells overexpressing CD36, SR-B1, or LOX-1 were maintained in Ham's F-12 medium containing 10% fetal calf serum and 0.5 mg/ml of G418 (medium A). For the uptake experiments, these cells were seeded in a 24-well plate (8×10^4/well) and cultured for 2 days in 1.0 ml of medium A, which was then replaced by medium B (Dulbecco's modified Eagle's medium containing 3% BSA). Human peripheral mononuclear cells were isolated from the blood of healthy volunteers by Ficoll density gradient centrifugation (Ficoll-Paque, Amersham Biosciences). The purified monocytes were suspended in RPMI 1640 and seeded in a 24-well plate (5×10^5 cells/well) and incubated for 1 h. The monolayers thus formed were washed three times with 1.0 ml of PBS. The cells were incubated for 7 days to differentiate into macrophages and then replaced by medium B for the uptake experiments [17]. The cells in each well were incubated at 37 °C for 8 h in 0.5 ml of medium B with three different concentrations of ^{125}I-high-AGE-BSA or ^{125}I-mild-AGE-BSA, in the presence or absence of their 50-fold unlabeled ligands. To measure the cell-associated radioactivity, each well was washed twice with 1.0 ml of 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 sodium hydroxide for 1 h at 37 °C to determine the cell-bound radioactivity and cellular proteins. The protein concentration was measured by bicinchoninic acid protein assay reagent (Pierce). The radioactivity of the sample was determined by gamma counter (ARC 360, Aloka, Tokyo, Japan). The specific association and degradation were determined by subtracting the nonspecific value from the total value [3].

2.6. Human lens samples

The human cataract lens samples were obtained after receiving the approval of the institutional review board of Shinshu University and Kumamoto University and obtaining informed consent from all patients. The fragments of lenses with cataract (8 from diabetes and 9 from non-diabetes) were collected while surgery, and then the protein concentration of the sample was measured with bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). The CML content of each sample was measured by an amino acid analysis as described above.

2.7. Clearance experiments

The experimental protocol was approved by the local ethics review committee for animal experimentation. The BSA, mild-AGE-BSA, and high-AGE-BSA were radiolabeled with ^{111}In using the bifunctional chelating reagent Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*‴-pentaacetic acid (DTPA) anhydride, according to the method of Hnatowich et al. [18]. The mice received tail vein injections of ^{111}In-labeled proteins in saline, at a dose of 1 mg/kg. At the appropriate intervals after the injection, blood was collected from the vena cava under anesthesia, and plasma was obtained by centrifugation. The organs were

excised, rinsed with saline and weighed. The radioactivity of each sample was measured in a well-type NaI scintillation counter (ARC-500, Aloka, Tokyo). In this study, the radioactivity of all samples and that of the plasma was normalized to the percentage of dose. The plasma concentrations were analyzed by a biexponential function using the non-linear least-squares computer program MULTI [19]. The two-compartment model was fitted according to the Akaike information criterion. The tissue distribution patterns were evaluated using tissue uptake clearances (CL_{uptake}) according to the integration plot analysis. CL_{uptake} was calculated by:

$$CL_{uptake} = \frac{X_t/C_t}{AUC_{0-t}/C_t}$$

where X_t is the tissue accumulation at time t, AUC_{0-t} is the area under the plasma concentration time-curve from time 0 to t, and C_t is the plasma concentration at time t. CL_{uptake} was obtained from the slope of the plot of X_t/C_t versus AUC_{0-t}/C_t. Each data point represents the mean±SD for three mice.

2.8. Statistical analysis

All of the experimental data are expressed as the mean±SD. The differences between groups were examined for statistical significance using either Student's *t*-test or one-way analysis of variance (ANOVA) with the Newman–Keuls *post-hoc* test. A P value less than 0.05 was considered to denote the presence of a statistically significant difference.

3. Results and discussions

3.1. Physicochemical properties of AGE-BSA

We first measured the increase in the molecular weight of AGE-BSA by a MALDI TOF mass analysis. As shown in Fig. 1A–C, the molecular mass of mild- and high-AGE-BSA were 658 Da and 9389 Da, respectively, and were larger than the native BSA. The net negative charge of AGE-BSA was determined by an agarose gel electrophoresis. The electrophoretic mobility of the high-AGE-BSA toward the anode was significantly higher than that of the unmodified BSA, whereas the mild-AGE-BSA slightly increased its mobility (Fig. 1D). Therefore, it is likely that the increase in the molecular weight and the negative charge of the high-AGE-BSA was strikingly higher than that of the mild-AGE-BSA. We next measured the reactivity of 6D12 to AGE-BSA. As shown in Fig. 1E, 6D12 significantly recognized both the high- and mild-AGE-BSA.

In the next step, the AGE-BSA and the human lens samples were subjected to HPLC to compare the content of CML, a major AGE structure, between the physiological samples and the AGE-BSA, which was prepared *in vitro*. For this purpose,

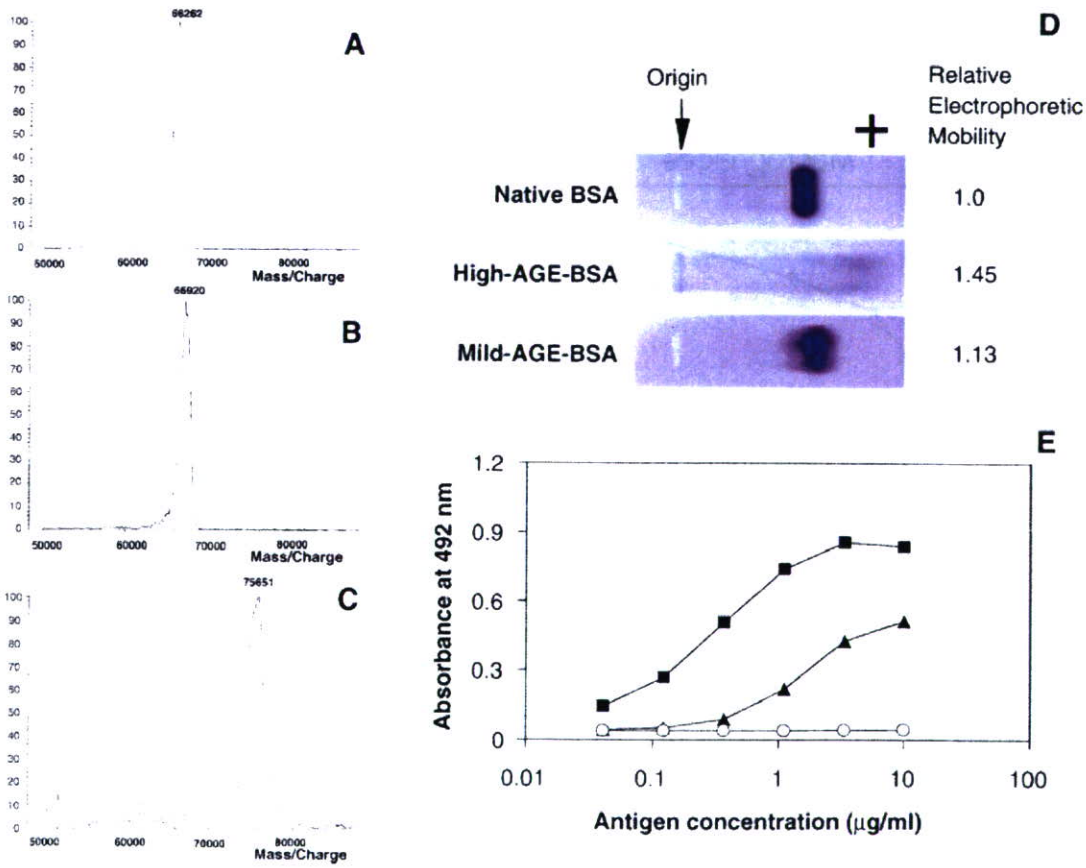

Fig. 1. Physicochemical properties of AGE-BSA. A MALDI-TOFMS analysis of native BSA (A), mild-AGE-BSA (B), and high-AGE-BSA (C). The molecular mass of the sample was analyzed by MALDI III mass spectrometer. (D) Agarose gel electrophoresis of AGE-BSA. Native BSA, mild-AGE-BSA, and high-AGE-BSA were subjected to agarose gel electrophoresis and stained with Coomassie brilliant blue. Their electrophoretic mobilities relative to native BSA were expressed as relative electrophoretic mobility. (E) The reactivity of mild- and high-AGE-BSA with 6D12 was determined by ELISA.

Table 1
Comparison of the CML content in the samples by HPLC

Samples	mmol CML/mol Lys
High-AGE-BSA	618.6
Mild-AGE-BSA	24.4
Diabetic lenses, $n=8$	17.4±3.8
Non-diabetic lenses, $n=9$	8.6±2.1

The samples were hydrolyzed with 6 N HCl for 24 h at 110 °C and then were subjected to HPLC. The amount of CML content was normalized to the lysine content of the sample. Data are mean±SD.

the human lens proteins were chosen because high amounts of AGE accumulation are observed in the long-lived proteins such as the lens proteins [20]. Table 1 summarizes the CML content in the samples. The CML content for the high- and mild-AGE-BSA were 618.6 mmol CML/mol Lys and 24.4 mmol CML/mol Lys, respectively. However, the CML contents for the diabetic- and non-diabetic human lens samples were ~17.4 mmol /mol Lys and ~8.6 mmol/mol Lys, respectively, thus indicating that our experimentally prepared mild-AGE-BSA was already more profoundly modified than physiological samples.

3.2. Ligand activity of AGE-BSA to human monocyte-derived macrophages and CHO cells overexpressing scavenger receptors

We next determined whether the high- and mild-AGE-BSA preparations could interact with cells as ligands. As shown in Fig. 2A, the ^{125}I-high-AGE-BSA was specifically associated in a dose-dependent manner, whereas these changes were not observed in the ^{125}I-mild-AGE-BSA. Similar tendencies were observed in the CHO overexpressing scavenger receptors. Therefore, the ^{125}I-high-AGE-BSA was specifically associated with the CHO cells overexpressing SR-BI (Fig. 2B), CD36 (Fig. 2C), and LOX-1 (Fig. 2D), whereas the association of the ^{125}I-mild-AGE-BSA to these cells was negligible, and thereby strongly demonstrated that only the high-AGE-BSA showed ligand activity to the scavenger receptors.

3.3. Plasma clearance and organ distribution of AGE-BSA

Fig. 3 shows the plasma clearance and organ distribution in the mice that had been intravenously injected with ^{111}In-BSA, ^{111}In-mild-AGE-BSA, and ^{111}In-high-AGE-BSA. The plasma clearance rates of ^{111}In-mild-AGE-BSA were very slow, similar to the ^{111}In-BSA, whereas the radioactivity of ^{111}In-high-AGE-BSA was rapidly cleared from the circulation, with about 90% of the injected ^{111}In-high-AGE-BSA being eliminated within 5 min after the intravenous administration (Fig. 3A). At 120 min after the intravenous injection of ^{111}In-high-AGE-BSA, the organ distribution was 60% for the liver, 1.5% for the spleen, and 4% for the kidney, suggesting that the rapid disappearance of the ^{111}In-high-AGE-BSA from the plasma is accompanied by a very pronounced increase in liver clearance (Fig. 3B–D). The uptake clearance calculated using the nonlinear least-squares

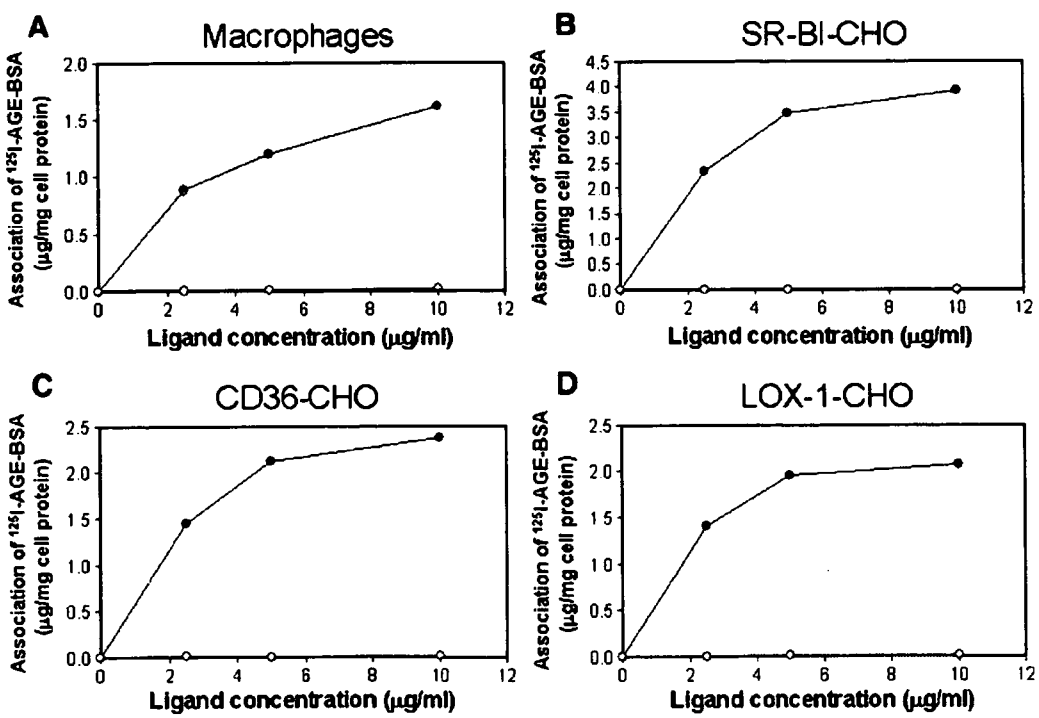

Fig. 2. Ligand activity of AGE-BSA to human monocyte-derived macrophages and CHO cells overexpressing scavenger receptors. The endocytic uptake of AGE-BSA by human monocyte-derived macrophages and CHO cells which overexpress CD36, SR-BI, and LOX-1. Human monocyte-derived macrophages (A), SR-BI-CHO (B), CD36-CHO (C), and LOX-1-CHO (D) cells were incubated at 37 °C for 8 h with the indicated concentration of ^{125}I-high-AGE-BSA (closed circles) or ^{125}I-mild-AGE-BSA (open circles) in the presence or absence of their 50-fold unlabeled ligands. The cells specific association of ^{125}I-high-AGE-BSA and ^{125}I-mild-AGE-BSA were calculated by correcting for nonspecific cell associations. Data are mean±SD, $n=2$.

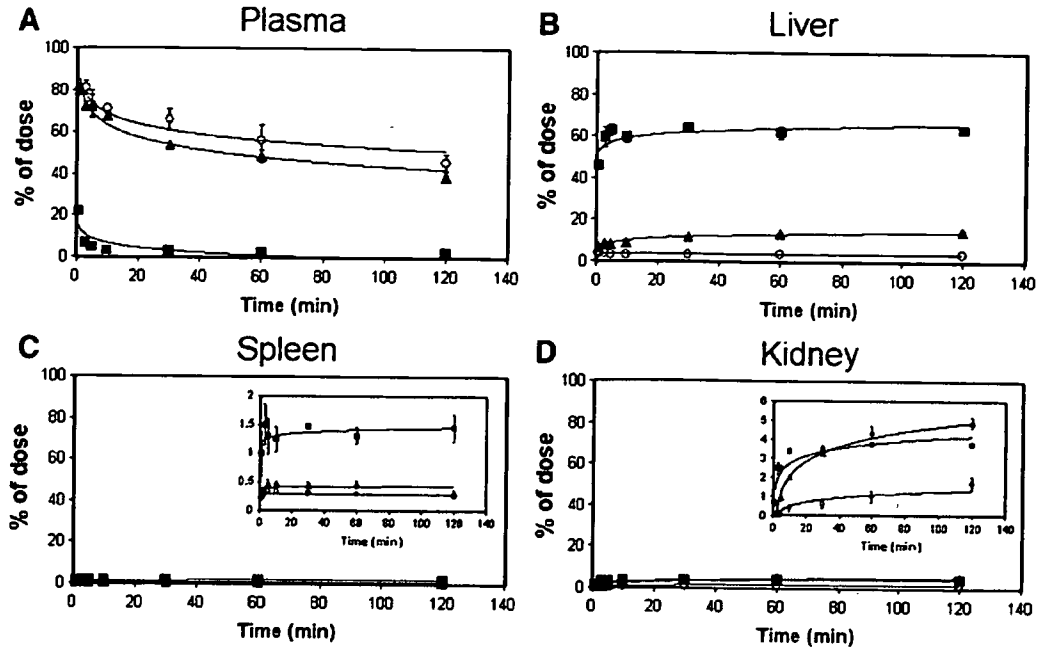

Fig. 3. Plasma clearance and organ distribution of AGE-BSA after intravenous administration to mice. ^{111}In-BSA (open circles), ^{111}In-mild-AGE-BSA (closed triangles), and ^{111}In-high-AGE-BSA (closed squares) were injected as a bolus through the tail vein of mice, and the relative radioactivities were plotted against the time after injection. Each data point represents the mean±SD for three mice.

computer program MULTI also indicated that the liver plays a major role in the clearance of high-AGE-BSA (Table 2). The accumulation by other organs such as the pancreas, lung, heart, and brain was negligibly low, and the pattern was indistinguishable from that of BSA. Since the scavenger receptors such as SR-A and SR-BI are highly expressed in the liver [21], it is reasonable to conclude that the high-AGE-BSA is endocytosed in the liver. In contrast, the clearance rate of the ^{111}In-mild-AGE-BSA was very slow in comparison to the ^{111}In-native BSA (Fig. 3A). Smedsrod et al. [22] demonstrated that intravenously injected 125I-high-AGE-BSA to rat rapidly accumulated in liver and about 60% of 125I-high-AGE-BSA was localized in endothelial cells. We also previously demonstrated that isolated mice liver endothelial cells recognize high-AGE-BSA as a ligand [23]. Those reports are correlated with our present results. Therefore, high-AGE-BSA was significantly recognized in the liver.

3.4. Correlation of the extents of lysine modification of glycolaldehyde-derived AGE-BSA with their ligand activities

Since we previously demonstrated that high-AGE-BSA and GA-AGE-BSA show the same ligand activity to the scavenger receptor [3], we next examined the correlation between the extent of the lysine modification of GA-AGE-BSA and its ligand activity to the scavenger receptor. For this purpose, we prepared the GA-AGE-BSA samples with different lysine modifications and the ^{125}I-high-AGE-BSA was incubated with RAW 264.7 cells in the presence of 50-fold unlabeled GA-AGE-BSA with different lysine modification samples. As shown in Fig. 4, although the association of ^{125}I-high-AGE-BSA to the cells was not inhibited in the presence of the 29% lysine-modified unlabeled GA-AGE-BSA, it was significantly inhibited in the presence of >49% lysine-modified unlabeled GA-AGE-BSA, demonstrating that high lysine-modification is required to be a ligand for the scavenger receptor. Furthermore,

Table 2
Uptake clearance of AGE-BSA after intravenous administration to mice

(μL/h)	Liver	Kidney	Spleen
BSA	57.5±13.1	44.4±1.9	12.6±4.8
Mild-AGE-BSA	273.3±53.8	199.1±33.0	21.9±11.6
High-AGE-BSA	35016.9±8554.4*,#	3185.3±689.6*,#	3983.8±964.4*,#

Data represent the mean±SD for three sets of experiments. *P< 0.001 versus BSA. #P<0.001 versus Mild-AGE-BSA.

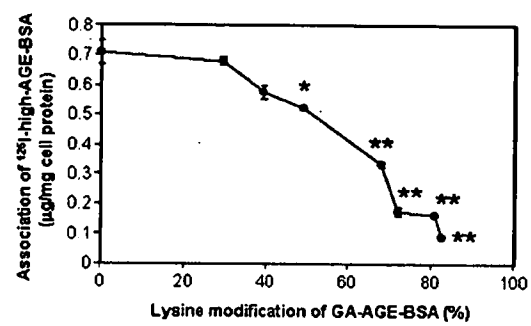

Fig. 4. Correlation of the extents of lysine modification of GA-AGE-BSA with their ligand activities. RAW 264.7 cells were incubated at 37 °C for 8 h with 1.25 μg/ml of ^{125}I-high-AGE-BSA in the presence of 50-fold unlabeled GA-AGE-BSA with different lysine modification samples. The cell association of ^{125}I-high-AGE-BSA was plotted against the extent of lysine modification of competitor GA-AGE-BSA. Data are mean±SD, n=2. *, P<0.05, **, P<0.01 vs. unmodified BSA.

as shown in Fig. 5, ^{125}I-GA-AGE-BSA was incubated with RAW 264.7 cells for 6 h in the presence of 50-fold excess amount of human lens samples. Although association of ^{125}I-GA-AGE-BSA was inhibited by unlabeled GA-AGE-BSA, mild-AGE-BSA and human lens samples did not show any inhibitory effect, thus indicating that physiological AGE-samples do not show any ligand activity to the scavenger receptors. Although we previously removed LPS from AGE-BSA by polylysine-conjugated sepharose column chromatography, we stopped removing LPS because the ligand activity of AGE-BSA to scavenger receptor does not change at all before and after removal of LPS. In fact, although association of ^{125}I-GA-AGE-BSA to RAW 264.7 cells was inhibited by unlabeled GA-AGE-BSA, the addition of 1–100 pg/ml of LPS did not show any inhibitory effect (Fig. 5). In the present study, we paid attention to using the fatty acid-free BSA instead of low-endotoxin BSA because Fu MX et al. [24] reported that CML is also generated from fatty acids such as arachidonate and linoleate, and thus may affect the ligand activity to scavenger receptors.

Immunohistochemical studies have demonstrated that GA-pyridine, one of the myeloperoxidase-derived AGEs through glycolaldehyde, was predominantly accumulated in the cytoplasm of the foam cells in human atherosclerotic lesions [25], thus indicating that GA-pyridine detected in foam cells may be generated within the cells. Regarding the AGE formation inside cells, Hammes et al. [26] reported that thiamine and benfotiamine prevent the intracellular AGE formation by reducing the concentration of methylglyoxal, a strong AGE-precursor, and hence inhibit diabetic retinopathy. In the case of the glycated-proteins presence *in vivo*, Thornalley et al. [15] demonstrated that end-stage renal disease is associated with a significant increase in the molecular mass of HSA (+255 Da, relative to the control subjects) and ~3% of lysine residues were modified. However, our study using a MALDI-TOFMS analysis demonstrated that the molecular mass of the mild-AGE-BSA was 658 Da larger than the native BSA (Fig. 1B), indicating that our experimentally prepared mild-AGE-BSA is already more profoundly modified than physiological human serum albumin under (patho)physiological conditions. Furthermore, although only ~3% of lysine residues of HSA were modified in dialysis patient [15], >49% lysine-modification is required to be a ligand for the scavenger receptor (Fig. 4). Taken together, our results demonstrate that the endocytic uptake of physiologically generated AGE-proteins through scavenger receptors is either negligible or unlikely to occur *in vivo*, and that AGEs detected inside foam cells in atherosclerotic lesions are generated intracellularly rather than representing the endocytic uptake of extracellular AGE-proteins by the scavenger receptors. Although most other researchers prepare the AGE-proteins with unphysiologically high concentrations of aldehydes and then are employed for the cellular experiments, some researchers consider the issues and have also prepared AGE-proteins with physiologically relevant concentrations of glycating agents in order to perform cellular experiments. For instance, macrophage-colony stimulating factor (M-CSF) is released from mature human monocytes and human monocytic THP-1 cells by incubating with HSA minimally-modified by methylglyoxal (MG_{min}-HSA) and glucose (AGE_{min}-HSA) [27]. MG_{min}-HSA also induces the synthesis and secretion of tumor necrosis factor-α (TNF-α) from human monocytic THP-1 cells in vitro [28]. Furthermore, Xu et al. [29] demonstrated that mildly-modified AGE-BSA which was prepared by incubating 50 mM glucose inhibits nitric oxide synthesis of human endothelial cells, indicating that several cellular interactions take place even AGE-proteins with physiologically relevant concentrations of glycating agents. These results suggest that mildly modified AGE-proteins may be recognized by AGE receptors such as RAGE and induce several cellular responses *in vivo*. This study demonstrates the first evidence that the ligand activity of the AGE-proteins to the scavenger receptors is dependent on their rate of modification by the AGEs, and we should carefully prepare the AGE-proteins *in vitro* to clarify the physiological significance of the interaction between the AGE-receptors and AGE-proteins.

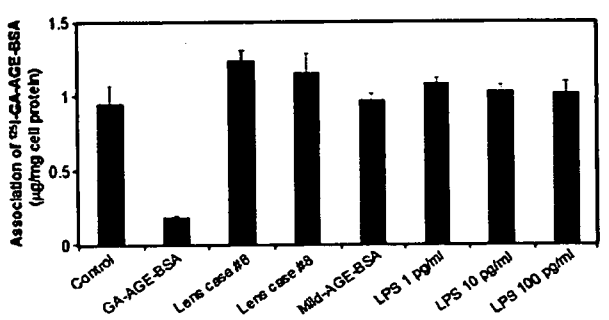

Fig. 5. Inhibitory effect of human lens proteins on the association of GA-AGE-BSA to RAW 264.7 cells. RAW 264.7 cells were incubated at 37 °C for 6 h with 2 μg/ml of ^{125}I-AGE-GA-BSA (82.5% of lysine residues were modified) in the presence of 50-fold unlabeled diabetic human lens samples, GA-AGE-BSA or mild-AGE-BSA. LPS was also used at 1–100 pg/ml. The CML content of diabetic lens samples case #6 and #8 were 19.6 and 16.8 mmol CML/mol Lys, respectively. Amounts of cell-associated ^{125}I-AGE-GA-BSA were determined as described in Materials and methods. Data are mean±SD, n=2.

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