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Renal clearance of glycolaldehyde- and methylglyoxal-modified proteins in mice is mediated by mesangial cells through a class A scavenger receptor (SR-A)

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Abstract *Aims/hypothesis:* Glomerular mesangial expansion is a characteristic feature of diabetic nephropathy, and the accumulation of AGE in the mesangial lesion has been implicated as one of its potential causes. However, the route for the AGE accumulation in mesangial lesions in diabetic patients is poorly established. *Methods:* Glycolaldehyde-modified BSA (GA-BSA) and methylglyoxal-modified BSA (MG-BSA) were prepared as model AGE proteins, and their in vivo plasma clearance was examined in mice, and renal uptake by in vitro studies with isolated renal mesangial cells. *Results:* Both ^{111}In-GA-BSA and ^{111}In-MG-BSA were rapidly cleared from the circulation mainly by both the liver and kidney. Immunohistochemical studies with an anti-GA-BSA antibody demonstrated that intravenously injected GA-BSA accumulated in mesangial cells, suggesting that such cells play an important role in the renal clearance of circulating AGE proteins. Binding experiments at 4°C using mesangial cells isolated from mice showed that ^{125}I-GA-BSA and ^{125}I-MG-BSA exhibited specific and saturable binding. Upon incubation at 37°C, ^{125}I-GA-BSA and ^{125}I-MG-BSA underwent endocytic degradation by these cells. The binding of the ligands to these cells was inhibited by several ligands for scavenger receptors. The endocytic degradation of GA-BSA by mesangial cells from class A scavenger receptor (SR-A) knock-out mice was reduced by 80% when compared with that of wild-type cells. The glomerular accumulation of GA-BSA after its intravenous administration was attenuated in SR-A knock-out mice, as evidenced by immunohistochemical observations. *Conclusions/interpretation:* These results raise the possibility that circulating AGE-modified proteins are subjected to renal clearance by mesangial cells, mainly via SR-A. This pathway may contribute to the pathogenesis of AGE-induced diabetic nephropathy.

Keywords AGE · Biological fate · Diabetic nephropathy · Endocytosis · Intermediate aldehydes · Scavenger receptor

Abbreviations Ac-LDL: Acetylated LDL · GA: Glycolaldehyde · MG: Methylglyoxal · Ox-LDL: Oxidized LDL · SR-A: Class A scavenger receptor

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Introduction

The long-term incubation of proteins with glucose leads, through the formation of early products such as Schiff bases and Amadori products, finally to AGE. AGE proteins are characterised physicochemically by their fluorescent, brown color and intramolecular and intermolecular cross-linking [1], and biologically by their recognition by specific AGE receptors [2]. Recent studies reported that several aldehydes such as glycolaldehyde (GA), glyoxal, methylglyoxal (MG), and 3-deoxyglucosone are generated during the Maillard reaction from glucose, a Schiff base, or Amadori products [3, 4]. A much stronger chemical reactivity than that of glucose indicates the important role of these aldehydes in the in vivo generation of AGE structures [5–7].

Previous studies have provided several lines of evidence for the pathological role of AGE in diabetic nephropathy. First, immunological studies using an anti-AGE antibody demonstrated the accumulation of AGE in glomerular mesangial lesions of diabetic nephropathy [8, 9]. Second, AGE proteins prepared by the modification of BSA with ribose,

induced TGF-β synthesis at an mRNA level in rat mesangial cells [10]. Third, AGE proteins prepared by incubating BSA with glucose-6-phosphate increased the levels of growth factors such as TGF-β and IGF-I and extracellular matrix proteins such as fibronectin, laminin and type IV collagen at the protein level in rat mesangial cells [11, 12]. Fourth, AGE proteins prepared by incubating BSA with glucose-6-phosphate also increased type IV collagen expression in human mesangial cells [13]. Finally, AGE proteins prepared by incubating BSA with glucose, glyceraldehyde, or GA induced the production of vascular endothelial growth factor and monocyte chemoattractant protein-1 in human mesangial cells [14].

These cellular responses to AGE proteins are believed to be mediated by AGE receptors which include the receptor for AGE (RAGE) [15], oligosaccharyltransferase-48 (OST-48), 80K-H, galectin-3 complex [16], and scavenger receptors such as the class A scavenger receptor (SR-A) [17], CD36 [18], SR-BI [19], and LOX-1 [20]. Several AGE receptor(s) in mesangial cells were characterised from the potential link to diabetic nephropathy [21–23]. However, one important issue that remains unknown concerns the access of AGE ligands to these AGE receptors in the mesangial area. More specifically, it is important to determine whether AGE proteins that accumulate in the mesangium are produced at the same site, or are produced in other places and then delivered to the mesangial area. We hypothesised that the increased levels of plasma AGE proteins in the diabetic states are subjected to renal clearance by an active cellular process(es) which may lead to the subsequent accumulation of AGE proteins in the mesangial area and induction of biological responses by interacting with AGE receptors of mesangial cells. In the present study, GA-modified BSA (GA-BSA) and MG-modified BSA (MG-BSA) were prepared as model AGE proteins and their plasma and renal clearance examined in mice. The endocytic uptake of these AGE proteins by mesangial cells separated from SR-A knock-out mice was also compared with that of control mice.

Materials and methods

Chemicals BSA (fraction V) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Penicillin G and streptomycin were purchased from Life Technologies (New York, NY, USA). RPMI 1640 medium, DMEM, Hanks' balanced salt solution (HBSS), biotinamidohexanoic acid *N*-hydroxysuccinimido ester (BNHS) and collagenase were obtained from Sigma Chemical (St Louis, MO, USA). ITS premix was obtained from Becton Dickinson (Bedford, MA, USA). Indium 111 trichloride (74 MBq/ml in 0.02 mol/l HCl) was a gift from Nihon Medi-Physics (Takarazuka, Japan), and $Na^{125}I$ (3.7 GBq/ml in NaOH) was purchased from Amersham Pharmacia Biotech (Little Chalfont, Bucks, UK). All reagents used were of the highest grade available from commercial sources.

Animals Male ddY (27–32 g) and C57BL/6 (22–25 g) mice were obtained from SLC (Shizuoka, Japan). The mice were kept under a 12/12 h light/dark cycle in a humidity-controlled room.

Mice lacking both SR-A were established from C57BL/6 by the targeted disruption of exon 4 of the type AI/type AII SR gene, which is essential for the formation of functional trimeric receptors in A3-1 embryonic stem cells. Descriptions of the construct and the phenotypic expression in homozygous knock-out mice have been reported previously [24]. Normal littermates were bred as controls.

Ligand preparation To prepare GA-BSA, 5 mg/ml BSA was incubated with 50 mmol/l of GA at 37°C for 3 days in PBS, followed by dialysis against PBS [3]. MG-BSA was prepared under identical conditions except that 50 mmol/l carbonate buffer (pH 9.0) was used instead of PBS. LDL (d=1.019–1.063 g/ml) was isolated by the sequential ultracentrifugation of fresh plasma from normolipidaemic subjects after overnight fasting, and dialysed against 0.15 mol/l NaCl per 1 mmol/l EDTA (pH 7.4) [25]. Acetylated LDL (Ac-LDL) was prepared by the chemical modification of LDL with acetic anhydride as described previously [25]. To prepare oxidized LDL (Ox-LDL), LDL was dialysed against PBS to remove EDTA. LDL (0.1 mg/ml) was then incubated with a 5 μmol/l solution of $CuSO_4$ at 37°C for 24 h, followed by the addition of 1 mmol/l EDTA and cooling.

Protein labelling with ^{111}In and ^{125}I BSA, GA-BSA, and MG-BSA were labelled with ^{111}In using diethylenetriaminepentaacetic acid (DTPA) as the bifunctional chelating agent as described previously [26]. DTPA was attached to BSA by dissolving the protein (5 mg) in 1.0 ml of 0.1 mol/l HEPES buffer (pH 7.0), and then adding 50 μg of DTPA anhydride in 10 μl of DMSO. After stirring for 1 h at room temperature, unreacted DTPA was removed by placing the solution on a Sephadex G-25 column (1×40 cm) followed by elution with 0.1 mol/l sodium acetate buffer (pH 6.0). Fractions containing DTPA-BSA were combined based on the absorption at 280 nm and concentrated by an ultrafiltration apparatus from Advantec (Dublin, CA, USA). Twenty microlitres of $^{111}InCl_3$ solution, 60 μl of DTPA-BSA, and 20 μl of 1 mol/l sodium acetate buffer (pH 6.0) were added. After incubation for 30 min at room temperature, unreacted $^{111}InCl_3$ was removed by adding the solution to a PD-10 column followed by elution with 0.1 mol/l sodium acetate buffer (pH 6.0). The ^{111}In-enriched fractions were selected on the basis of their radioactivity and concentrated by ultrafiltration. Radioactivity was determined with a well counter. The specific activities of ^{111}In-BSA, ^{111}In-GA-BSA, and ^{111}In-MG-BSA were 12×10^6, 11×10^6 and 11×10^6 cpm/mg protein, respectively.

GA-BSA and MG-BSA were labelled with ^{125}I by Iodo-Gen (Pierce). A solution containing 10 μl of $Na^{125}I$ solution and 0.5 mg of BSA in 0.2 ml of 0.1 mol/l sodium phosphate buffer (pH 7.4) in a Iodo-Gen-adhered test tube was incubated for 30 min at room temperature. Unreacted $Na^{125}I$ was removed by adding the solution to a PD-10 column followed by elution with PBS. The ^{125}I-labelled fractions

were combined on the basis of their radioactivity, as determined with a well counter. The specific activities of GA-BSA and MG-BSA were 800 and 900 cpm/ng protein, respectively.

Clearance experiments Prior to intravenous administration, trace amounts of ^{111}In-BSA, ^{111}In-GA-BSA, or ^{111}In-MG-BSA were diluted with saline, and the protein concentration was adjusted to 0.1 mg/ml. Each radiolabeled protein (0.1 mg/kg, 10 kcpm) was injected as a bolus into the tail vein of male ddY mice at 7 weeks of age after being anaesthetised with diethylether [27]. After 1, 3, 5, 10, 15, or 20 min, the animals were killed, and the kidney and the liver were excised, followed by a determination of their radioactivities for kidney and liver clearance. To determine the organ distribution of each ^{111}In-labelled protein, the animals were similarly killed at 20 min after injection, and organs such as kidney, liver, pancreas, spleen, lung, heart, and brain were excised to determine their radioactivity. Care and treatment of the animals was in compliance with the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985). The protocol was approved by the University of Kumamoto institutional review board for animal procedures.

Data analyses In this study, the radioactivity of liver and kidney samples was used to calculate the apparent uptake clearances of BSA, GA-BSA, and MG-BSA. The radioactivity of all samples and that of the plasma was normalised to the percentage of dose or percentage of dose per millilitre, respectively. Plasma concentrations were analysed by a biexponential function using the non-linear least-squares computer program MULTI [28]. The two-compartment model was chosen according to the Akaike information criterion.

Total body clearance of radioactivity (CL_{total}) was calculated by:

$$CL_{total} = \frac{D}{AUC}$$

where D is the dose of radioactivity administered, and AUC is the area under the plasma concentration–time curve extrapolated to infinity.

Assuming zero or negligible leakage of radioactivity from organs, the apparent organ uptake clearance (CL_{organ}) may be expressed as:

$$CL_{organ} = \frac{X_{ti}}{AUC_{t0-ti}}$$

where X_{ti} is the amount of radioactivity in the organ of interest at the end of the experiment, and AUC_{t0-ti} is the area under the curve for the time interval from t0 = administration of the compound, to ti = end of the experiment.

Preparation of biotinylated antibody against GA-BSA The monoclonal anti-GA-BSA antibody was prepared and purified by protein G-immobilised Sepharose gel chromatography to IgG1, as described previously [5]. This monoclonal antibody was shown to be specific for the glycolaldehyde-derived pyridine (GA-pyridine) structure [5] and is referred to as the anti-GA-pyridine antibody in the present study [5]. The anti-GA-pyridine antibody (0.5 mg) was biotinylated by reaction with 50 μg of BNHS for 1 h at room temperature in 0.1 mol/l carbonate buffer (pH 8.4) and dialysed against PBS to remove unreacted BNHS. The immunoreactivity of the biotinylated antibody for GA-BSA was indistinguishable from the unlabelled anti-GA-pyridine antibody as determined by ELISA.

Immunohistochemistry GA-BSA (5 mg/kg) was injected as a bolus into the tail vein of male C57BL/6 mice at 7 weeks of age after being anaesthetised with diethylether. After 5 min, the animals were killed, and the kidneys removed, fixed with a 2% periodate/lysine/paraformaldehyde fixative at 4°C for 5 h and washed with PBS containing a graded series of sucrose (10, 15 and 20%). After immersion in PBS containing 20% sucrose to inhibit ice crystal formation, the tissues were embedded in OCT compound (Sakura Fine Technical, Tokyo, Japan), frozen in liquid nitrogen, and stored at −80°C until used. Sections were cut 5-μm thick with a cryostat (HM-500 M; Microm, Walldorf, Germany) and mounted on poly-L-lysine-coated slides.

For immunohistochemical analysis, cryostat sections were prepared and examined by the indirect immunoperoxidase method. Briefly, after inhibition of endogenous peroxidase activity by the method of Isobe et al. [29], the sections were incubated with 10 μg/ml of biotinylated anti-GA-pyridine antibody, washed with PBS, and reacted with streptavidin/HRP (DAKO). After visualisation with 3,3′-diaminobenzidine (Dojin Chemical, Kumamoto, Japan), the sections were counterstained with haematoxylin (Mutoh Chemical, Tokyo, Japan). For negative controls, the identical procedures were performed, but the antibody and the biotinylated antibody were omitted. Non-immune mouse IgG1 was also used as a negative control, and staining showed no evidence for an immunoreaction. For the immunoabsorption test, 10 μg/ml of biotinylated anti-GA-pyridine antibody was preincubated with 10 μg/ml of GA-BSA at 37°C for 1 h and allowed to react with streptavidin/HRP.

Cellular experiments with mesangial cells Isolation of glomerular cores was performed by the method of Mori et al. [30] with a minor modification. Briefly, the kidneys of male ddY mice at 7 weeks of age were removed under anaesthesia with diethylether and cut into slices. Each slice was pressed through a 425-μm-pore-size sieve. The pass-through was rinsed three times in HBSS and pellets were centrifuged at 3,000 rpm for 10 min. The pellets were then incubated for 30 min at 37°C with 750 U/ml of collagenase in 5 ml of HBSS, followed by washing three times with HBSS for centrifugation and passing through two sieves with pore sizes of 100 and 40 μm. The glomerular cores remaining on the finest sieve were cultured at 37°C for 2

weeks in RPMI 1640 medium with 20% FCS, 100 U/ml penicillin, 10 μg/ml streptomycin, and ITS premix in 75-cm^2 plastic tissue culture flasks in a 5% CO_2 incubator. Mesangial cells obtained from the fifth to eighth passage were plated on 12-well plates at 7.5×10^4 cells/well and cultured for 24 h at 37°C in RPMI 1640 medium with 20% FCS, 100 U/ml penicillin, and 10 μg/ml streptomycin before each experiment described below. Except for the binding experiments described below, all cellular experiments were performed at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cultured mesangial cells used in the present study formed a uniform cell population based on the following criteria: (1) spindle shape; (2) positive immunohistochemistry for both β-actin (Fig. 1a) and vimentin (Fig. 1b), but negative for cytokeratin (Fig. 1c) and Factor VIII (Fig. 1d); (3) contraction in response to angiotensin II and vasopressin; and (4) induction of TGF-β by serotonin [31].

An additional note to the method: although our modified method—sieving of glomerular cores after collagenase treatment—gives a high yield of glomerular cores and subsequent mesangial cells, special attention has to be paid to the possibility that incubation with collagenase could lead to the overdigestion of glomerular cores and a low yield of glomerular core on the finest sieve. To prevent this, the readers are highly recommended to do preliminary experiments to determine the adequate incubation time for collagenase treatment as well as selection of the suitable collagenase batch from commercially available ones.

For binding experiments, confluent mesangial cells were washed twice with 1.0 ml of PBS and incubated for 90 min at 4°C with 1.0 ml of DMEM supplemented with 3% (w/v) BSA containing the indicated concentrations of ^{125}I-GA-BSA or ^{125}I-MG-BSA with or without a 50-fold excess of unlabelled GA-BSA or MG-BSA. After washing three times with ice-cold PBS containing 1% (w/v) BSA and twice further with PBS, the cells were dissolved in 1.0 ml of 0.1 mol/l NaOH, and cell-bound radioactivity and cellular proteins were determined.

For uptake and degradation experiments, confluent cells were washed twice with PBS and incubated at 37°C with 1.0 ml of DMEM supplemented with 3% (w/v) BSA containing the indicated concentrations of ^{125}I-GA-BSA or ^{125}I-MG-BSA with or without a 50-fold excess of unlabelled GA-BSA or MG-BSA. After incubation for the indicated time intervals, culture medium (0.75 ml) was taken from each well and mixed with 0.3 ml of 40% trichloroacetic acid on a vortex mixer. To this solution was

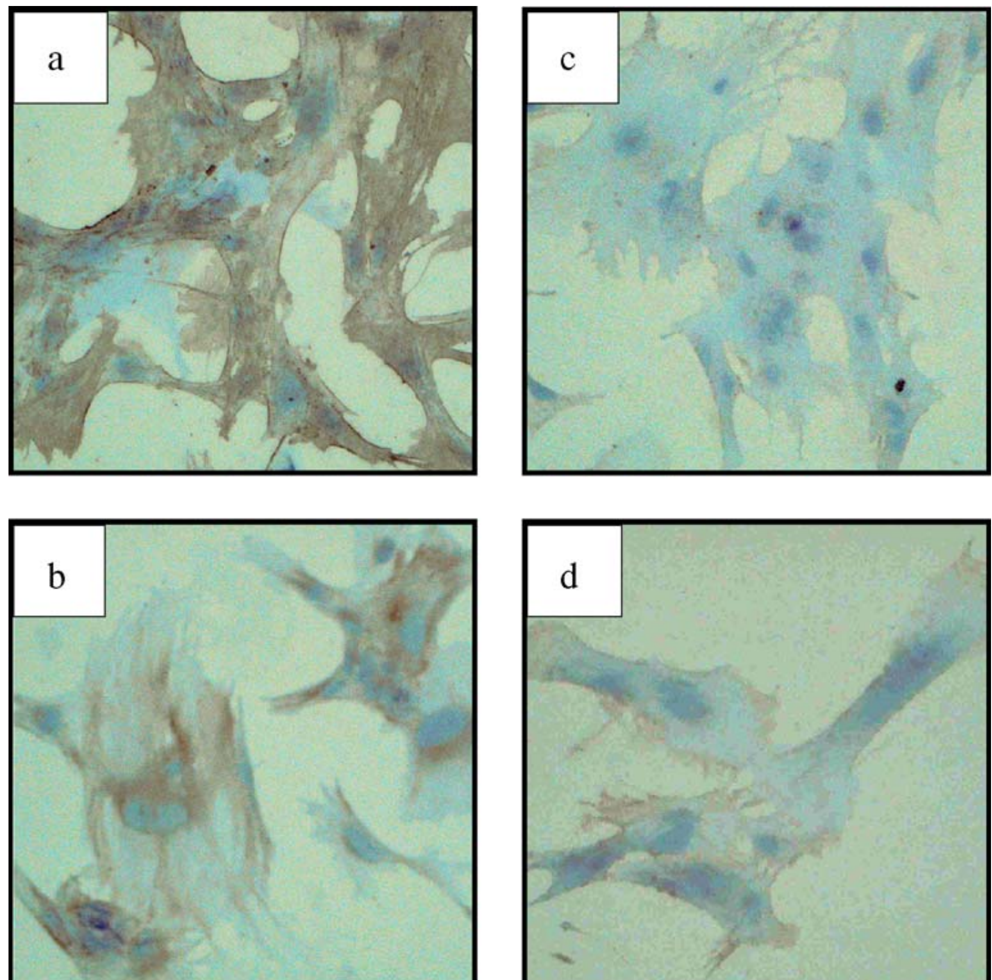

Fig. 1 Immunohistochemical staining for β-actin (**a**), vimentin (**b**), cytokeratin (**c**) and Factor VIII (**d**) in mouse mesangial cells. Staining of mouse mesangial cells between the fifth and eighth passages was positive for β-actin (**a**) and vimentin (**b**), but negative for cytokeratin (**c**) and Factor VIII (**d**)

added 0.2 ml of 0.7 mol/l $AgNO_3$, followed by centrifugation. The resulting supernatant was used to determine trichloroacetic acid-soluble radioactivity, which was taken as an index of cellular degradation, since AGE proteins are endocytosed by the cells and delivered to lysosomes where they are degraded and excreted into the culture medium in a trichloroacetic acid-soluble form. The remaining cells in each well were washed three times with 1.0 ml of PBS containing 1% (w/v) BSA and twice more with PBS. The cells were lysed at 37°C for 30 min with 1.0 ml of 0.1 mol/l NaOH. One portion was used to determine the radioactivity of the cell-associated ligand and the other was used to determine cellular proteins using the bicinchoninic acid protein assay reagent (Pierce) [32].

For inhibition assays, mesangial cells were incubated at 4°C for 90 min with 2.5 μg/ml of ^{125}I-GA-BSA or ^{125}I-MG-BSA in the absence (control) or presence of 40-fold (100 μg/ml) of unlabelled ligands such as GA-BSA, MG-BSA, Ox-LDL, Ac-LDL, and LDL. The extents of cell-bound ^{125}I-GA-BSA and ^{125}I-MG-BSA were determined as described above.

Statistical analysis Data are expressed as mean ± SD. Differences between groups were evaluated by paired Student's *t*-test. A *p* value less than 5% denoted a statistically significant difference.

Results

Plasma clearance of GA-BSA and MG-BSA Figure 2 shows the plasma clearance in mice that had been intravenously injected with ^{111}In-BSA, ^{111}In-GA-BSA, and ^{111}In-MG-BSA; the clearance rate of ^{111}In-BSA was very slow, whereas the radioactivity of ^{111}In-GA-BSA or ^{111}In-MG-BSA was rapidly cleared from the circulation, with about 80% of the injected ^{111}In-GA-BSA or ^{111}In-MG-BSA being eliminated within 5 min after intravenous administration. Figure 3 shows tissue distribution of ^{111}In-BSA, ^{111}In-GA-BSA, and ^{111}In-MG-BSA. At 20 min after the injection of ^{111}In-GA-BSA, 38% of the total injected radioactivity accumulated in the liver, and its renal accumulation corresponded to 21% (Fig. 3b). A similar pattern was obtained for ^{111}In-MG-BSA, with 32% for hepatic accumulation and 19% for renal accumulation (Fig. 3c). Accumulation by other organs such as pancreas, spleen, lung, heart, and brain was negligibly low, and the pattern was indistinguishable from that of BSA (Fig. 3a).

The clearance rates for liver and kidney were also calculated using the nonlinear least-squares computer program MULTI (Table 1). These CL_{liver} and CL_{kidney} values for GA-BSA and MG-BSA were significantly larger than those for BSA, suggesting the presence of a selective uptake system for GA-BSA and MG-BSA in both the liver and kidney.

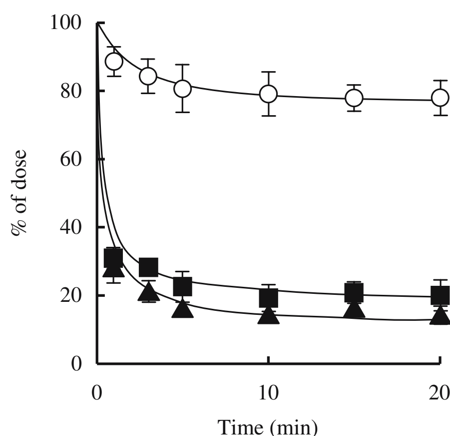

Fig. 2 Plasma clearance of ^{111}In-BSA, ^{111}In-GA-BSA, and ^{111}In-MG-BSA after intravenous administration to mice; ^{111}In-BSA (*open circles*), ^{111}In-GA-BSA (*closed triangles*), and ^{111}In-MG-BSA (*closed squares*) were injected as a bolus through the tail vein of mice, and the relative radioactivities are plotted against time after injection. Each data point represents the mean ± SD for three mice

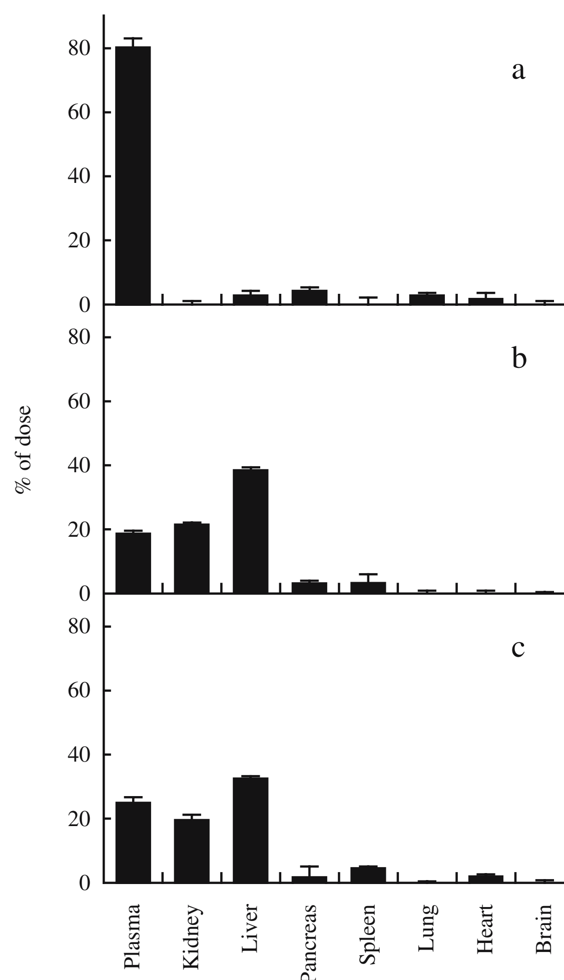

Fig. 3 Anatomical distribution of intravenously injected ^{111}In-BSA (**a**), ^{111}In-GA-BSA (**b**), and ^{111}In-MG-BSA (**c**). The mice used for plasma clearance studies (Fig. 2) were analysed for anatomical distribution of radioactivity 20 min after the injection. Approximately 90% of the injected dose was recovered in the tissues listed in all experiments. Results are means ± SD for three separate experiments

Table 1 Uptake of ^{111}In-BSA, ^{111}In-GA-BSA and ^{111}In-MG-BSA by mice liver and kidney

	Clearance (μl/h)		
	CL_{total}	CL_{liver}	CL_{kidney}
BSA	57.2±18.1	6.3±3.2	6.9±2.7
GA-BSA	16,573.8±1,173.5	9,123.5±603.2	3,767.2±481.6
MG-BSA	11,615.6±869.2	5,546.3±251.3	2,505.3±338.2

Values are means ± SD of three sets of experiments

Immunohistochemical distribution in kidney of intravenously injected GA-BSA To determine the cells responsible for renal clearance of AGE proteins from the circulation, the distribution of intravenously administered GA-BSA in the kidney was examined immunohistochemically using the biotinylated anti-GA-pyridine antibody as described in "Materials and methods." At 5 min after an intravenous injection of GA-BSA, positive immunoreactivities were found in the glomerular area with mesangial cells and endothelial cells being the main cellular components (Fig. 4a). These positive immunoreactions in the glomerulus were completely inhibited when the biotinylated antibody was pretreated with 10 μg/ml of GA-BSA (Fig. 4c). Furthermore, no positive immunoreactions were found in the renal glomerulus when the mice were subjected to an intravenous injection of BSA (Fig. 4d). It seems likely from these immunohistochemical data that mesangial cells, at least, are responsible for the renal clearance of intravenously administered GA-BSA.

GA-BSA and MG-BSA undergo receptor-mediated endocytosis by mesangial cells To determine the mechanism for the selective accumulation of intravenously injected GA-BSA and MG-BSA in the mesangium, we examined the cellular interaction of GA-BSA and MG-BSA using isolated mesangial cells. The total binding of ^{125}I-GA-BSA to these cells at 4°C was inhibited by >80% by the presence of an excess amount of unlabelled GA-BSA (Fig. 5a). The specific binding, which was obtained by subtracting non-specific binding from the total binding, showed a saturation curve for which Scatchard analysis revealed a binding site with an apparent Kd of 6.93 μg/ml and a maximal binding of 94.11 ng/mg of cell protein (Fig. 5a, inset). The specific binding of MG-BSA to mesangial cells was also saturable (Fig. 5b), the Scatchard analysis of which gave a binding site with an apparent Kd of 6.80 μg/ml and a maximal surface binding of 27.69 ng/mg of cell protein

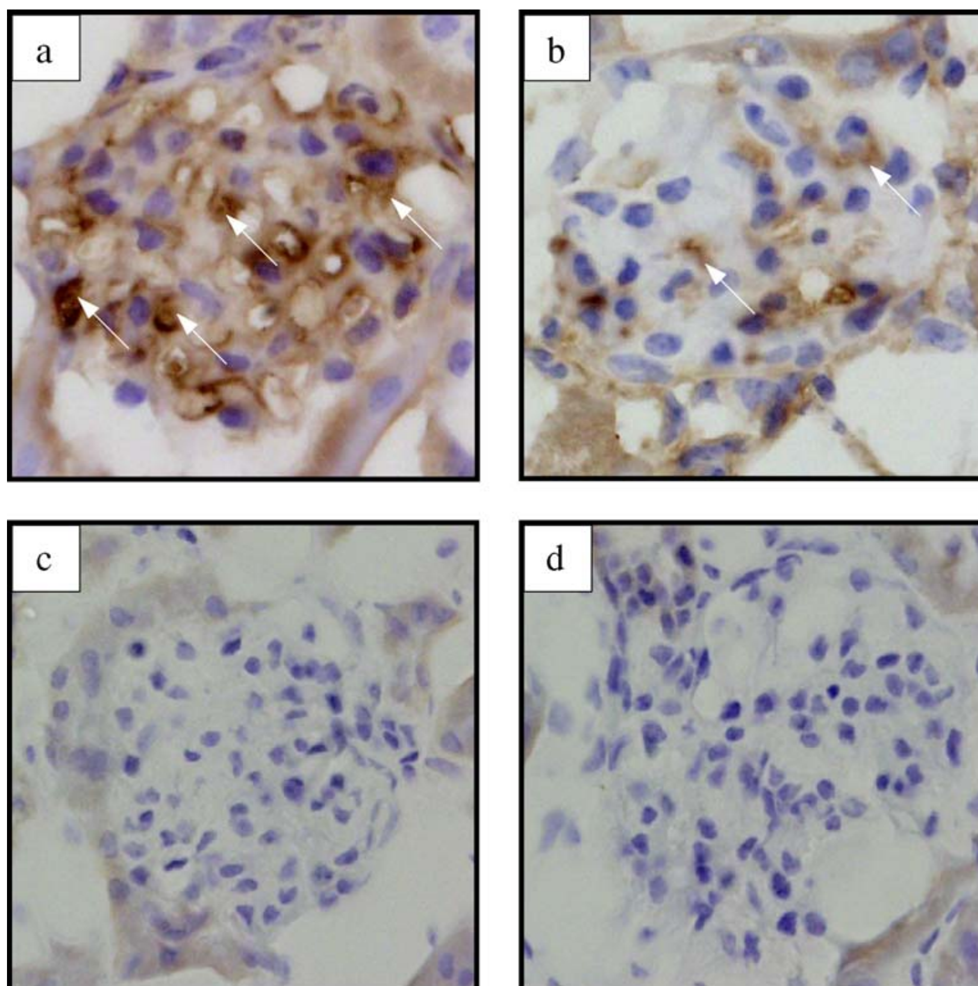

Fig. 4 Immunohistochemical localisation in kidney of GA-BSA after its intravenous administration to mice. The kidney was removed 5 min after an injection of GA-BSA or BSA in the form of a bolus through the tail veins of mice. **a** Anti-GA-pyridine antibody-positive products were observed in mesangial cells and endothelial cells in glomerulus obtained from mice that had been administered GA-BSA (*arrows*). **b** Positive reactions for anti-GA-pyridine antibody in glomerular areas observed in (**a**) were significantly weakened in SR-A knock-out mice. **c** Such positive reactions disappeared after pre-incubation of the antibody with 10 μg/ml of GA-BSA. **d** No immunoreactivity of anti-GA-pyridine antibody was detected in the glomerulus obtained from mice administered BSA

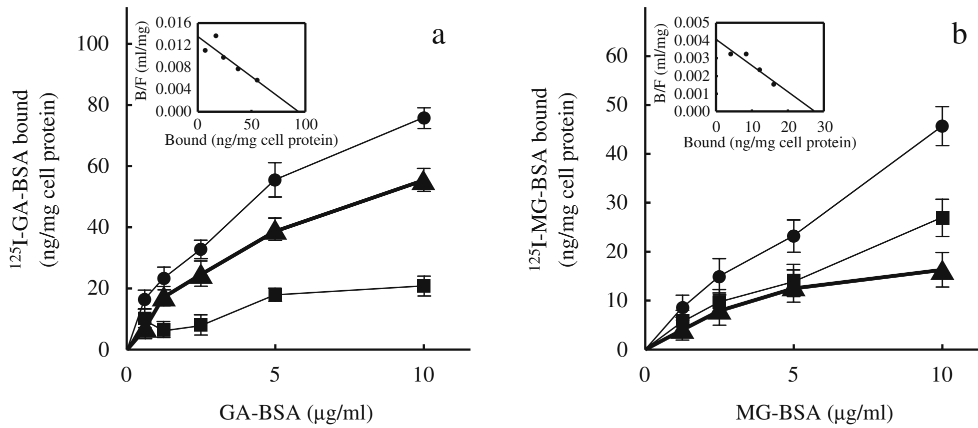

Fig. 5 Binding of ^{125}I-GA-BSA (**a**) and ^{125}I-MG-BSA (**b**) to mouse mesangial cells. Mesangial cells were incubated at 4°C for 90 min with the indicated concentrations of ^{125}I-GA-BSA (**a**) or ^{125}I-MG-BSA (**b**) in the presence (*closed squares*) or absence (*closed circles*) of 50-fold unlabelled ligands. *Closed triangles* indicate the specific binding of each ligand. *Inset* Scatchard analysis based on specific binding results for GA-BSA (**a**) and MG-BSA (**b**) to mesangial cells. Results are the means ± SD of three separate experiments

(Fig. 5b, inset). These results indicate that mesangial cells possess a high-affinity binding site for GA-BSA and MG-BSA.

To determine the post-binding outcome of GA-BSA and MG-BSA, these mesangial cells were incubated with ^{125}I-GA-BSA and ^{125}I-MG-BSA at 37°C. The amount of cell-associated ^{125}I-GA-BSA increased with time (Fig. 6a) followed by a parallel increase in the amount of ^{125}I-GA-BSA degraded by these cells (Fig. 6b). In a similar fashion, the amount of ^{125}I-MG-BSA associated with (Fig. 6c) and those degraded by these cells (Fig. 6d) increased with time. Based on these experiments, it appears likely that mesangial cells express receptors that are specific for GA-BSA and MG-BSA, and that ligands that bind to these receptors undergo receptor-mediated endocytosis.

To characterise the receptor for GA-BSA and MG-BSA on mesangial cells, we tested the effects of several ligands for scavenger receptors, such as Ac-LDL and Ox-LDL. The binding of ^{125}I-GA-BSA was effectively inhibited not only by unlabelled GA-BSA but also by MG-BSA, Ac-LDL, and Ox-LDL, whereas LDL showed no significant effect (Fig. 7a). Similarly, the binding of ^{125}I-MG-BSA was effectively inhibited by unlabelled GA-BSA, MG-BSA, Ac-LDL, and Ox-LDL, but not by LDL (Fig. 7b). These results suggest

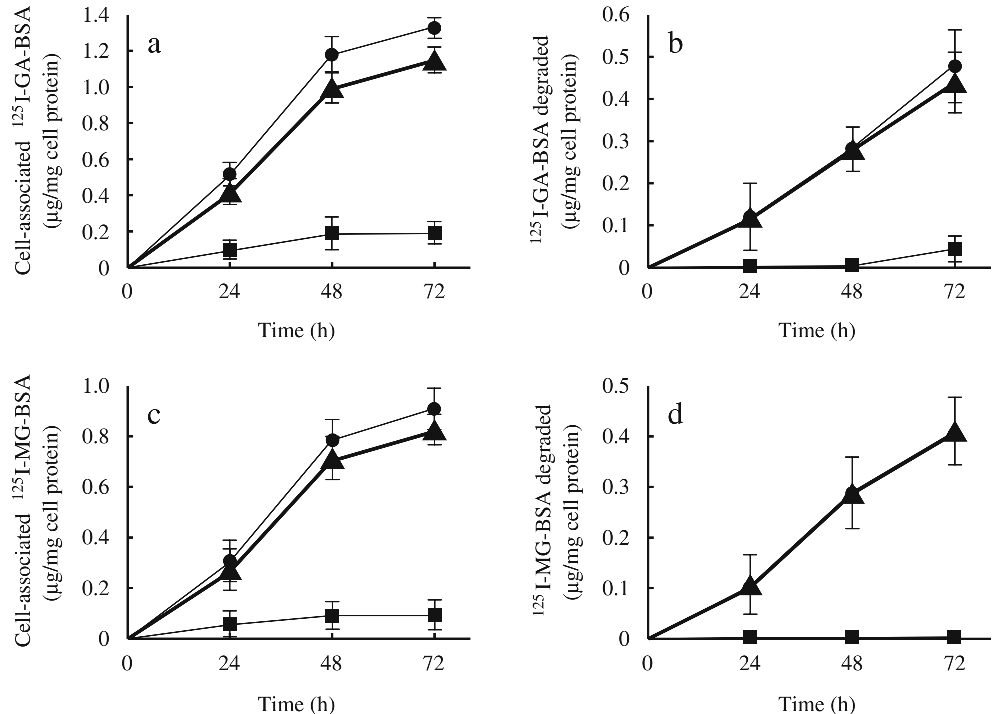

Fig. 6 Time-dependent endocytic uptake (**a**, **c**) and degradation (**b**, **d**) by mouse mesangial cells of ^{125}I-GA-BSA (**a**, **b**) and ^{125}I-MG-BSA (**c**, **d**). Mesangial cells were incubated at 37°C for the indicated times with 5 μg/ml of ^{125}I-GA-BSA or ^{125}I-MG-BSA in the presence (*closed squares*) or absence (*closed circles*) of 50-fold unlabelled ligands. *Closed triangles* indicate the specific association or degradation of each ligand. The amounts of cell-associated ^{125}I-GA-BSA and ^{125}I-MG-BSA and their degradation were determined as described in Materials and methods. Results are the means ± SD of three separate experiments

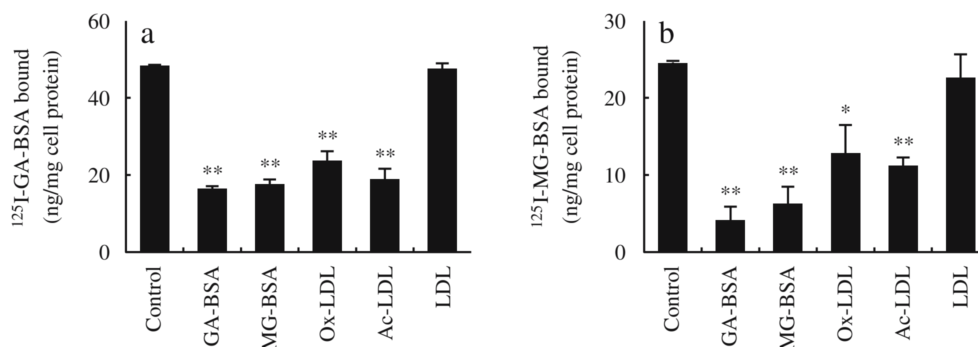

Fig. 7 Effects of several ligands on binding to mouse mesangial cells of ^{125}I-GA-BSA (**a**) and ^{125}I-MG-BSA (**b**). Mesangial cells were incubated at 4°C for 90 min with 2.5 μg/ml of ^{125}I-GA-BSA or ^{125}I-MG-BSA in the absence (control) or the 40-fold presence of unlabelled ligands such as GA-BSA, MG-BSA, Ox-LDL, Ac-LDL, and LDL. The extent of cellular binding of ^{125}I-GA-BSA and ^{125}I-MG-BSA was determined as described in Materials and methods. Results are the means ± SD of three separate experiments; **p*<0.05, ***p*<0.01, compared with the control

that GA-BSA and MG-BSA are recognised by mesangial cells via a scavenger receptor(s) and undergo receptor-mediated endocytosis.

Endocytic uptake of GA-BSA by mesangial cells from SR-A knock-out mice Among the scavenger receptors, SR-A [17], CD36 [18], SR-BI [19], and LOX-1 [20] have been identified as the AGE receptors. In this study we determined the extent of contribution of SR-A to the endocytic degradation of GA-BSA by mesangial cells. The specific binding of ^{125}I-GA-BSA to mesangial cells from SR-A knock-out mice was reduced by 37%, compared with that of wild-type mesangial cells. The extent of subsequent endocytic degradation of ^{125}I-GA-BSA by mesangial cells from SR-A knock-out mice was reduced by more than 80%, compared with those of wild-type cells (Fig. 8). These results suggest that SR-A probably plays a major role in the endocytic degradation of GA-BSA by mesangial cells.

To elucidate whether the renal uptake of circulating AGE proteins is mediated through the SR-A pathway in vivo, immunohistochemical experiments with kidneys after the injection of GA-BSA to SR-A knock-out mice were undertaken. Positive reactions were observed in mesangial cells and endothelial cells in wild-type mice (Fig. 4a), but the same reactions in SR-A knock-out mice were less (Fig. 4b), suggesting the glomerular accumulation of GA-BSA from the circulation via SR-A in vivo.

Discussion

The findings herein show that GA-BSA and MG-BSA leave the circulation rapidly and accumulate in the liver

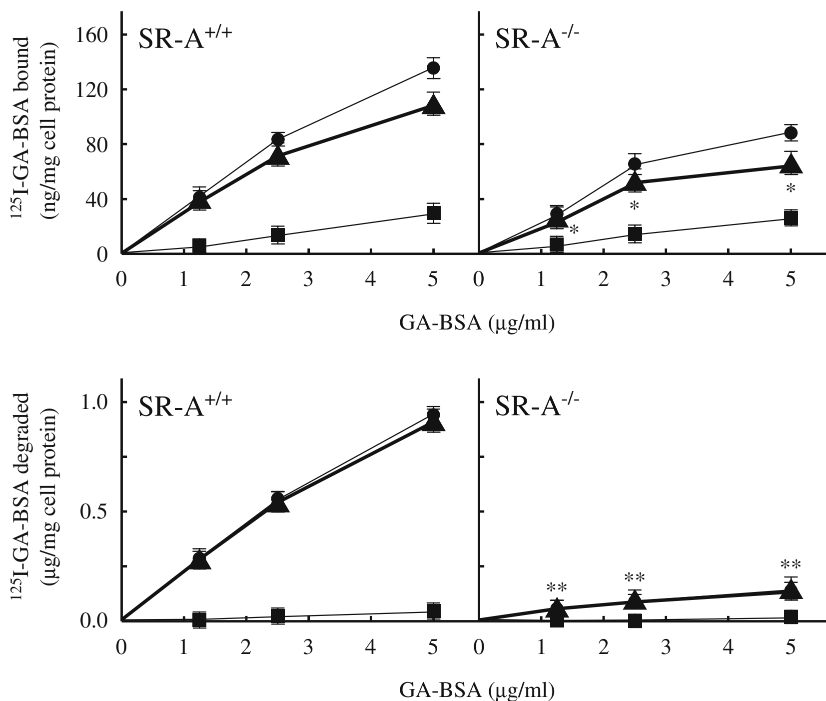

Fig. 8 Binding to, and endocytic degradation by, mesangial cells from SR-A knock-out mice of ^{125}I-GA-BSA. Mesangial cells obtained from SR-A knock-out mice (*right*; SR-A$^{-/-}$) and their littermates (*left*; SR-A$^{+/+}$) were incubated at 4°C for 90 min (*upper panels*) or 37°C for 72 h (*lower panels*) with the indicated concentrations of ^{125}I-GA-BSA in the presence (*closed squares*) or absence (*closed circles*) of 50-fold unlabelled ligands. *Closed triangles* indicate the specific binding or degradation of ^{125}I-GA-BSA. Results are the means ± SD of three separate experiments; **p*<0.05, ***p*<0.01, compared with the corresponding concentration for the specific binding and endocytic degradation of SR-A$^{+/+}$

and kidney (Fig. 3). Schmidt et al. [33] prepared AGE-BSA by incubating BSA with 250 mmol/l glucose-6-phosphate at 37°C for 4 weeks and reported that its plasma clearance in mice was rapid with 70% of the injected dose being removed from the circulation within 5 min. The amount of organ distribution at 10 min after intravenous injection was 43% for the liver, 17% for the lung, and 7% for the kidney, suggesting that the liver was the main organ for plasma clearance, but the removal by lungs and kidneys was not negligible. Vlassara et al. [34] prepared AGE rat serum albumin (RSA) by incubating RSA with 50 mmol/l glucose-6-phosphate at 37°C for 6 weeks, and its daily intravenous injection for 5 months (25 mg/kg per day) resulted in the induction of glomerular hypertrophy under which renal AGE accumulation was 1.5-fold higher than the controls, suggesting that intravenously administered AGE proteins could be trapped by the kidney in vivo. The results of the present study consistently showed that intravenously injected ^{111}In-labelled GA-BSA or MG-BSA in mice led to a significant renal accumulation of these ligands: 21% for GA-BSA and 19% for MG-BSA at 20 min after injection. However, data reported by Smedsrød et al. [35] are somewhat different: AGE-BSA prepared by incubating BSA with 1.7 mol/l of glucose for 40 weeks at 37°C was labelled with ^{125}I and its hepatic and renal accumulation determined after intravenous injection; the hepatic accumulation was dominant (94%) whereas the renal accumulation of ligands was very low (2.4% of the total injected radioactivity) at 60 min after an intravenous administration in the rat. It is not clear why the extent of renal accumulation after an intravenous injection of these AGE ligands differs from one experiment to another. It could reflect a difference in conditions used in preparing AGE proteins. Alternatively, the difference could be due to the method used for protein labelling. The present study used ^{111}In-labelled ligands whereas previous studies had used ^{125}I-labelled ligands. Proteins labelled with ^{111}In are known to have several advantages over those labelled with ^{125}I. The exchange of ^{111}In from ^{111}In-labelled proteins to other proteins in the lysosome results in a better target retention of radioactivity, whereas an ^{125}I-labelled protein can be degraded in lysosomes and released outside the cells, and the resulting amount of ^{125}I radioactivity accumulated in tissues could be underestimated [27].

For the accumulation of intravenously injected GA-BSA in the kidney, an immunoreaction for the anti-GA-pyridine antibody was found in the mesangial and endothelial cell areas (Fig. 4a). In this context, previous immunohistochemical studies using anti-AGE antibodies (mainly anti-CML antibodies) revealed the accumulation of AGE proteins in the mesangial lesions in diabetic nephropathy [8, 9]. In addition, an immunohistochemical study demonstrated the presence of SR-A on mesangial cell membranes in renal biopsy specimens from patients with glomerular diseases [21]. RT-PCR analyses revealed the expression of AGE receptors including OST-48, 80 K-H, and galectin-3 complex in mesangial cells that had been isolated from both normal and diabetic mice, and binding experiments using AGE-BSA with these cells revealed a specific binding with an apparent Kd of 160–300 nmol/l [22]. Binding experiments using AGE-BSA with membrane fractions isolated from rat and human mesangial cells revealed a saturable binding with a Kd of 500 nmol/l [36]. Finally, from the present study, it is clear that mouse mesangial cells are able to efficiently endocytose and degrade GA-BSA and MG-BSA (Figs. 5 and 6). Collectively, these data indicate that it is likely that mesangial cells are responsible for the renal uptake of AGE proteins from the circulation.

The present result shows that GA-BSA and MG-BSA bind to mouse mesangial cells, and that this binding is effectively inhibited by Ox-LDL and Ac-LDL as well as by MG-BSA, but not by LDL (Fig. 7). This indicates that the ligand specificity of the receptor involved is similar to that of scavenger receptors rather than that of RAGE, OST-48, 80 K-H, and galectin-3 complex. Consistent with this observation, a previous study showed that ^{125}I-Ox-LDL was degraded by rat mesangial cells, and that this process was effectively inhibited by unlabelled Ac-LDL, suggesting the involvement of scavenger receptor(s) [37]. Four scavenger receptors have been identified as AGE receptors: SR-A [17], CD36 [18], SR-BI [19], and LOX-1 [20]. Since a previous immunohistochemical study using an anti-SR-A antibody showed the presence of SR-A in human mesangial cells [21], and we also confirmed the expression of SR-A mRNA in mouse mesangial cells (data not shown), the contribution of SR-A to the endocytic uptake of these AGE proteins by mesangial cells was assessed in the present study. Our experiments with mesangial cells from SR-A knock-out mice indicate that SR-A contributes ~40% to the binding and ~80% to the subsequent degradation of AGE-modified proteins. The difference in contribution of SR-A to binding and degradation is not clear at present, but might be explained by (1) the expression of other AGE receptors such as RAGE, OST-48, 80 K-H, and galectin-3 complex in mesangial cells [22, 23] and (2) the fact that these receptors do not belong to the endocytosis-coupled receptor (ligand binding to these receptor does not follow the subsequent endocytic uptake, in sharp contrast to SR-A).

A recent study using SR-A knock-out mice has clarified the crucial role of SR-A in the pathogenesis of diabetic nephropathy; diabetic wild-type mice showed typical signs of diabetic nephropathy such as an increase in urinary albumin excretion, glomerular hypertrophy, an increase in mesangial matrix and fibrosis in interstitial tissues, whereas these pathologies were effectively ameliorated in diabetic SR-A knock-out mice (Shikata et al., personal communication).

In summary, the earliest morphological abnormalities in diabetic nephropathy are the thickening of the glomerular basement membrane and the expansion of mesangial lesions due to the accumulation of extracellular matrix [38]. The interaction of AGE-modified proteins with mesangial cells led to an increased production of collagen, fibronectin, laminin, and proteoglycan [12, 13]. Therefore, the present study provides new evidence indicating that circulating AGE-modified proteins are cleared from the kidney by SR-

A-mediated endocytic uptake by mesangial cells, suggesting that AGE proteins delivered by this pathway might play some role in the pathogenesis of diabetic nephropathy.

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