Endocytic Uptake of Nonenzymatically Glycosylated Proteins Is Mediated by a Scavenger Receptor for Aldehyde-modified Proteins

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Long term incubation of proteins with glucose, named the Maillard reaction (Maillard, L. C. (1912) C. R. Acad. Sci. (Paris) 154, 66-68), gives rise to advanced glycosylation end product (AGE) with fluorescence, color, as well as cross-linked properties. The receptor-mediated endocytosis of AGE-proteins by macrophages was studied (Vlassara, H., Brownlee, M., and Cerami, A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5588-5592). The present study on the binding of AGE-bovine serum albumin (BSA) to rat peritoneal macrophages and sinusoidal liver cells demonstrated the presence of a saturable, high affinity receptor for AGE-BSA with Kd = 2.4 × 10^-7 M (macrophages) and 2.1 × 10^-7 M (sinusoidal cells). The cellular binding of AGE-BSA and its endocytic uptake by these cells were competitively inhibited by BSA preparations modified with aliphatic aldehydes such as formaldehyde or glycolaldehyde, ligands known to be specific for a scavenger receptor for aldehyde-modified proteins (Horiiuchi, S., Murakami, M., Takata, K., and Morino, Y. (1986). J. Biol. Chem. 261, 4962-4966). These ligands also had a profound in vivo effect on the plasma clearance of AGE-BSA as well as its hepatic uptake. Thus, endocytic uptake of AGE-proteins by macrophages appeared to be mediated by a scavenger receptor for aldehyde-modified proteins. This provides evidence for the biological importance of the scavenger receptor in eliminating senescent macromolecules from the circulation.

Receptor-mediated endocytosis by macrophages or macrophage-derived cells of chemically modified proteins termed collectively as “scavenger function” has been studied in view of its potential link to atherosclerosis (1-4) and diabetic complications or aging processes (5, 6). Based on the fact that these ligands are avidly taken up by sinusoidal liver cells upon intravenous administration, we have focused on the molecular mechanism of the scavenger function of these cells. Our previous studies have shown that f-Alb was taken up by sinusoidal liver cells by a receptor-mediated mechanism (7, 8) and that the receptor involved was different from that mediating the endocytic uptake of acetylated low density lipoprotein (9). Subsequent studies on its ligand specificity showed that proteins modified with formaldehyde as well as with other aliphatic aldehydes such as glycolaldehyde, glycaldehyde, and propionaldehyde were recognized by this receptor as effective ligands (10, 11). We thus proposed the possibility that, although it was discovered originally as being specific for f-Alb (12, 13), the f-Alb receptor could play as a general scavenger receptor for aldehyde-modified proteins.

However, one of the critical questions on this issue which has remained unanswered is what the physiological ligand for this receptor in vivo could be. In this connection, Vlassara et al. (14) reported an interesting observation. When albumin was subjected to long term incubation with glucose known as the Maillard reaction (15), the advanced glycosylation end product (AGE) of BSA exhibited a specific interaction with mouse peritoneal macrophages: AGE-BSA (AGE-modified BSA prepared by long term incubation with glucose) underwent receptor-mediated endocytosis by these cells. They also demonstrated that the binding of 125I-AGE-BSA to these cells was inhibited by BSA coupled covalently to FFI. Since FFI was claimed to be one of the major senescent fluorescent compounds produced in vitro as well as in vivo by the advanced stage of the Maillard reaction (16, 17), this observation drove them to consider that FFI might serve as a signal for the receptor-mediated recognition of senescent proteins (14).

The purpose of the present study was 3-fold: to determine whether AGE-proteins indeed undergo the receptor-mediated endocytosis by rat peritoneal macrophages and by sinusoidal liver cells, to assess the role of FFI in this biological recognition, and to examine the possibility that the receptor for AGE-BSA is identical or similar to the f-Alb receptor. The results indicate that AGE-proteins such as AGE-BSA, AGE-human plasma albumin, and AGE-human hemoglobin act as effective ligands for the scavenger receptor for aldehyde-modified proteins. It is suggested therefore that AGE-proteins might serve as natural ligands for this scavenger receptor. However, FFI portions of AGE-proteins are not involved in the biological recognition.

MATERIALS AND METHODS

Chemicals—BSA (Fraction V), human serum albumin, and human hemoglobin were purchased from Sigma. BSA was chromatographed on a Sephasryl S-200 column, and the monomeric fraction was used as an albumin source for preparation of ligands. Formaldehyde, glycolaldehyde, β-glucose, and collagenase were purchased from Wako Chemical Co. (Osaka, Japan). Sodium 125I (15.8 mCi/μg iodine) was obtained from Du Pont-New England Nuclear. Other chemicals were of the best grade available from commercial sources.

Preparation of AGE-Proteins and Aldehyde-modified Proteins—
AGE-BSA, AGE-human serum albumin, and AGE-human hemoglobin were prepared as described (14, 16, 17). BSA (2 g), human serum albumin (0.5 g), and human hemoglobin (0.5 g) were incubated in 10 ml of 0.5 M sodium phosphate buffer (pH 7.4) with 3 g of glucose at 37 °C for 60 days under aseptic conditions, followed by dialysis against 20 ml of 0.15 M NaCl. A portion of each AGE-protein was incubated with 50 mM NaCNBH₃ or NaBH₄ at 37 °C for 4 h, followed by dialysis against 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl. All AGE-proteins thus prepared showed the absorption and fluorescent spectra identical to those reported previously (16). To examine the effect of reduction by NaCNBH₃ or NaBH₄ on the generation of the ligand activity, a parallel incubation was performed in the presence of 50 mM NaCNBH₃ or NaBH₄. A portion of each AGE-protein was incubated with 50 mM NaCNBH₃ or NaBH₄ at 37 °C for 4 h, followed by dialysis against 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl.

f-Alb, glycol-Alb, and glycol-Hb were prepared as described (10, 11). Briefly, BSA was incubated in 0.1 M sodium carbonate buffer (pH 10.0) with 0.33 M formaldehyde at 37 °C for 1 h, or 50 mM glycolaldehyde at 37 °C for 6 h. Parallel incubations were performed in the presence of 50 mM NaCNBH₃ or NaBH₄. These samples were dialyzed as described above. Portions of f-Alb and glycol-Alb thus prepared were further treated with 50 mM NaCNBH₃ or NaBH₄ (10). The protein was determined by the modified method (18) of Lowry et al. (19) with BSA as a standard.

Conjugates-Two different FFI-BSA conjugates were prepared. FFI-HA and FFI-BA were coupled to BSA by the methods reported previously (16, 17). Chemical structures were confirmed by 1H NMR spectroscopy. Details will appear elsewhere.²

Preparation of FFI-BSA Conjugates—Two different FFI-BSA conjugates were prepared. FFI-HA and FFI-BA were coupled to BSA with water-soluble carbodiimide. Briefly, to FFI-HA (10 mg, 30 μmol) or FFI-BA (10 mg, 32 μmol) dissolved in 3 ml of acetonitrile/distilled water (10:90) was added 1-ε-cyclohexyl-3-(2-4-morpholinyl)ethyl carbodiimide (10 mg, 23 μmol). After a 10 min-incubation at room temperature, 1 ml of BSA (20 mg/ml) in water was added, followed by overnight incubation. After dialysis against distilled water and then 10 mM sodium phosphate buffer containing 0.15 M NaCl (pH 7.2), glycolated BSA thus prepared were confirmed by 1H NMR spectroscopy. Details will appear elsewhere.³

Chemical Synthesis of FFI and Its Derivatives — 2-(2-Furyl)-4-(2-furanyl)-1H-imidazole (FFI), 4-furanyl-2-furyl-1H-imidazole-1-hexanoic acid (FFI-HA), and 4-furanyl-2-furyl-1H-imidazole-1-butyric acid (FFI-BA) were synthesized with some modifications of the method previously (15, 16). Chemical structures were confirmed by 1H NMR spectroscopy. Details will appear elsewhere.³

Preparation of FFI-BSA conjugates—Two different FFI-BSA conjugates were prepared. FFI-HA and FFI-BA were coupled to BSA with water-soluble carbodiimide. Briefly, to FFI-HA (10 mg, 30 μmol) or FFI-BA (10 mg, 32 μmol) dissolved in 3 ml of acetonitrile/distilled water (10:90) was added 1-ε-cyclohexyl-3-(2-4-morpholinyl)ethyl carbodiimide (10 mg, 23 μmol). After a 10 min-incubation at room temperature, 1 ml of BSA (20 mg/ml) in water was added, followed by overnight incubation. After dialysis against distilled water and then 10 mM sodium phosphate buffer containing 0.15 M NaCl (pH 7.2), glycolated BSA thus prepared were confirmed by a brief centrifugation. Amounts of FFI derivatives incorporated into BSA were estimated by spectrophotometric measurement on the basis of the molar extinction coefficient for FFI (εmax = 9664) (14). The values were 7.6 mol/mol BSA for FFI-HA-BSA and 5.7 mol/mol BSA for FFI-BA-BSA.

Ligand oxidation—All ligands were radiolabeled with ¹²⁵I with Iodo-Gen (Bio-Rad) (20) and dialyzed overnight at 4 °C against 20 mM sodium phosphate buffer (pH 7.4). Each labeled ligand was >98% trichloroacetic acid-precipitable. Specific radioactivities for AGE-BSA, f-Alb, and glycol-Hb were prepared as described (10, 12). The values of triplicate assays. The Scatchard analyses were performed as described above.

RESULTS

In Vivo Studies—Plasma clearance was measured as described previously (10). Buffer A containing 4.2 μg of ¹²⁵I-AGE-BSA was injected intravenously (0.1 ml/100 g of body weight) via the femoral vein of a male Wistar rat (200-250 g). Blood samples (0.1 ml) were withdrawn at various times from the jugular vein into heparinized tubes, followed by centrifugation at 1000 X g for 5 min at 4 °C. Plasma samples were measured for trichloroacetic acid-soluble radioactivity. The amount of acid-soluble radioactivity was expressed as a percentage of the injected dose, assuming a plasma volume of 3.13 ml/100 g body weight as described (10). To examine the effect of unlabeled ligands on the plasma clearance, 1.5 mg of AGE-BSA, f-Alb, or glycol-Alb was simultaneously injected. Plasma clearance of ¹²⁵I-f-Alb (6.6 μg) was studied as described for ¹²⁵I-AGE-BSA.

To examine effects of unlabeled ligands on the hepatic uptake of AGE-BSA, 8.3 μg of ¹²⁵I-AGE-BSA was injected with or without 3 mg of unlabeled AGE-BSA or f-Alb. At 10 min after injection via the femoral vein, the liver was flushed out through the portal vein with 200-250 ml of cold 0.15 M NaCl (pH 7.2) and then the supernatant (0.3 ml) was taken with 1 ml of 10% trichloroacetic acid to determine trichloroacetic acid-soluble radioactivity as described (21). Free iodine was extracted with chloroform after oxidation with hydrogen peroxide (22). The cell-associated radioactivity was also determined after washing the cells with buffer A as described above.

Intracellular Binding—All ligands were radiolabeled with ¹²⁵I with Iodo-Gen (Bio-Rad) (20) and dialyzed overnight at 4 °C against 20 mM sodium phosphate buffer containing 0.15 M NaCl (pH 7.4). Each labeled ligand was >98% trichloroacetic acid-precipitable. Specific radioactivities for AGE-BSA, f-Alb, and glycol-Hb were prepared as described (10, 12). The values of triplicate assays. The Scatchard analyses were performed as described above.

Effect of FFI on the Binding Process—Vlassara et al. (14) observed that cell surface-bound FFI-AGE-BSA was endocytosed and delivered to lysosomes for degradation.

specific binding as described under "Materials and Methods." After incubation on ice for 1 h in the absence of (0) or presence (●) of 0.9 mg/ml unlabeled AGE-BSA, the cell-associated radioactivity was measured. The nonspecific binding was <10% of the total binding. The specific binding (△) was plotted after correction for nonspecific binding as described under "Materials and Methods." B, peritoneal macrophages adhered to each culture dish were incubated in 0.5 ml of buffer A at 37°C. At intervals, cells were sedimented by brief centrifugation. Trichloroacetic acid-soluble radioactivity in the supernatant (●) and the cell-associated radioactivity (●) were determined as described under "Materials and Methods." Each tube contained, in a total volume of 0.1 ml of buffer A, a total volume of 0.1 ml of buffer A was injected simultaneously. B, rats were injected with 4.8 μg of 125I-FFI-HA-BSA to determine the plasma clearance rates for FFI derivatives. Effects of AGE-BSA (●) and FFI-HA-BSA (□) were determined in the same way by simultaneous injection of 1.5 mg of each unlabeled ligand. To test whether this was the case with our system, we synthesized FFI and its derivatives and studied the effects of FFI and FFI-HA conjugates on the cellular binding of 125I-AGE-BSA to sinusoidal cells as well as to macrophages. However, no significant competition was observed with any of these FFI compounds (Fig. 3). This finding was inconsistent with the notion that the FFI structure of AGE-BSA might serve as a receptor-binding domain (14). Furthermore, the plasma clearance of 125I-FFI-BSA was very slow as observed with unmodified BSA, and it was not affected by simultaneous injection of an excess of the same unlabeled ligand (Fig. 4B). When 125I-AGE-BSA was injected intravenously into rats, it disappeared very rapidly with a half-life of a few minutes. The clearance was markedly retarded by the simultaneous injection of a loading dose of AGE-BSA, while it was not affected by injection of FFI-BSA (Fig. 4A). Such in vivo behavior of 125I-FFI-BSA was fully supported by the finding that it did not exhibit any sign of the specific cellular binding either to sinusoidal cells or macrophages (Fig. 5). Thus, the FFI structure may not be involved in the receptor-

FIG. 1. Binding of 125I-AGE-BSA to rat sinusoidal liver cells and peritoneal macrophages. A, each tube contained 2×10⁶ cells and indicated concentrations of 125I-AGE-BSA in 0.1 ml of buffer A. After incubation on ice for 1 h in the absence of (0) or presence (●) of 0.9 mg/ml unlabeled AGE-BSA, the cell-associated radioactivity was measured. The nonspecific binding was <10% of the total binding. The specific binding (△) was plotted after correction for nonspecific binding as described under "Materials and Methods." B, peritoneal macrophages adhered to each culture dish were incubated in 0.5 ml of buffer A at 37°C for 2 h with graded amounts of 125I-AGE-BSA in the presence of (●) or absence (○) of 1.5 mg/ml unlabeled AGE-BSA. The cells were then washed to determine the cell-associated radioactivity. The specific binding (△) was determined as described above. The Scatchard analysis of each specific binding curve is shown in the inset.

FIG. 2. Endocytic uptake of 125I-AGE-BSA by sinusoidal liver cells and peritoneal macrophages. A, each tube containing 2×10⁶ cells and 7 μg/ml of 125I-AGE-BSA in 0.1 ml of buffer A was incubated at 37°C. At the indicated times, cells were sedimented by brief centrifugation. Trichloroacetic acid-soluble radioactivity in the supernatant (●) and the cell-associated radioactivity (●) were determined as described under "Materials and Methods." B, each well was seeded with 2×10⁶ peritoneal macrophages and was incubated with 9 μg/ml 125I-AGE-BSA in 0.5 ml of buffer A at 37°C. At intervals, both the amount of trichloroacetic acid-soluble radioactivity (○) and the amount of cell-accumulated radioactivity (●) were determined.

FIG. 4. Plasma clearance of 125I-AGE-BSA and 125I-FFI-BSA. A, rats were injected intravenously with 5.2 μg of 125I-AGE-BSA, and their plasma clearance rates were determined (●). Under parallel conditions, 1.5 mg of unlabeled AGE-BSA (○) or FFI-HA-BSA (□) was injected simultaneously. B, rats were injected with 4.8 μg of 125I-FFI-HA-BSA to determine the plasma clearance rates (●). Effects of AGE-BSA (●) and FFI-HA-BSA (□) were determined in the same way by simultaneous injection of 1.5 mg of each unlabeled ligand.

Fig. 3. Effect of FFI-derivatives on binding of 125I-AGE-BSA to sinusoidal liver cells. Each tube contained, in a total volume of 0.1 ml of buffer A, 2×10⁶ cells and 7.1 μg/ml of 125I-AGE-BSA in the presence of indicated concentrations of unlabeled FFI-HA-BSA (●), FFI-Ba-BSA (□) or AGE-BSA (○). After incubation at 4°C for 1 h, the amounts of the cell-associated radioactivity were determined as described under "Materials and Methods." The 100% value determined in the absence of any competing compounds was 3.4 ng/10⁶ cells. FFI per se had no effect on this binding process up to 20 μM (data not included).
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FIG. 5. Binding of $^{125}$I-FFI-BSA to sinusoidal liver cells. The cells ($2 \times 10^4$) were incubated on ice for 1 h with varying amounts of $^{125}$I-FFI-HA-BSA in 0.1 ml of buffer A with or without 1 mg/ml unlabeled FFI-HA-BSA. The total binding ($\square$), nonspecific binding ($\circ$), and specific binding ($\triangle$) were determined as described under "Materials and Methods."

FIG. 6. Effect of AGE-BSA and f-Alb on their endocytic uptake by macrophages. A, macrophages adhered to each well were incubated at 37°C for 4 h in 0.5 ml of buffer A with 14 $\mu$g/ml $^{125}$I-AGE-BSA. After extensive washing of cells, the cell-associated radioactivity was determined. Effect of unlabeled ligands on the uptake of $^{125}$I-AGE-BSA was tested in parallel incubations with 0.5 mg/ml each of AGE-BSA, f-Alb, glycol-Alb, and glycol-Hb. B, the parallel incubations were made as described for A, except that $^{125}$I-AGE-BSA was replaced by 10 $\mu$g/ml $^{125}$I-f-Alb. Unlabeled ligands tested were the same as those in A. Assays were run in quadruplicate. The 100% values for $^{125}$I-AGE-BSA and $^{125}$I-f-Alb were 0.88 and 0.1 $\mu$g/mg cell protein, respectively.

mediated recognition of AGE-BSA.

Effect of Aldehyde-modified Proteins on Receptor-mediated Recognition of AGE-BSA—It is known that intravenous injection of chemically modified proteins such as acetylated low density lipoprotein and aldehyde-modified albumin results in rapid endocytic uptake of these ligands via scavenger receptors on sinusoidal liver cells (7-9, 24, 25). Since the in vivo behavior of AGE-BSA appeared to be very similar to that of ligands for the scavenger receptors, we have examined the effect of various ligands on the cellular uptake of $^{125}$I-AGE-BSA. As shown in Fig. 6A, the uptake of $^{125}$I-AGE-BSA by peritoneal macrophages was inhibited similarly by the aldehyde-modified proteins such as f-Alb, glycol-Alb, and glycol-Hb. The uptake of $^{125}$I-f-Alb by macrophages was also inhibited efficiently by AGE-BSA as well as by f-Alb, glycol-Alb, or glycol-Hb (Fig. 6B). Similar results were obtained from experiments using sinusoidal liver cells: the cellular uptake of $^{125}$I-f-AGE-BSA was inhibited by f-Alb and AGE-BSA, and the uptake of $^{125}$I-f-ALb was also inhibited by AGE-BSA and f-Alb in a dose-related manner (Fig. 7). The results of these cross-competition experiments strongly suggest that AGE-BSA is recognized by a receptor identical or closely similar to a receptor which also recognizes f-Alb, glycol-Alb, and other aldehyde-modified proteins.

Cross-competition Experiments between AGE-BSA and f-Alb in Their Plasma Clearance—The plasma clearance of $^{125}$I-AGE-BSA was significantly retarded when a loading amount of unlabeled f-Alb or glycol-Alb was injected simultaneously (Fig. 8A). When a similar study was done with $^{125}$I-f-Alb, its plasma clearance rate was retarded both by unlabeled AGE-BSA as well as f-Alb or glycol-Alb to a similar extent (Fig. 8B). Thus, it is likely that both AGE-BSA and f-Alb might be cleared from the circulation by the same mechanism, probably by the same scavenger receptor system. Since the scavenger receptor for the aldehyde-modified proteins is concentrated on sinusoidal liver cells (10, 11), the hepatic uptake systems for $^{125}$I-AGE-BSA and $^{125}$I-f-Alb were compared in
cross-competition experiments. Within 10 min after injection via the femoral vein, ~57% of the total $^{125}$I-AGE-BSA administered was accumulated into the liver. This accumulation level was decreased by >85% upon simultaneous injection of a loading amount of unlabeled AGE-BSA or f-Alb (Fig. 9A). The hepatic uptake of $^{125}$I-f-Alb within 10 min postinjection (~58% of the total dose) was also inhibited to a similar degree (>80%) by a loading amount of AGE-BSA or f-Alb (Fig. 9B).

Some Ligand Specificity of the Receptor for AGE-BSA—To obtain some information about the ligand specificity of the receptor for AGE-BSA, AGE-human serum albumin and AGE-human hemoglobin were tested for their effect on the cellular uptake of $^{125}$I-AGE-BSA and $^{125}$I-f-Alb. As shown in Table I, they exhibited a significant inhibition on the endocytic uptake of $^{125}$I-AGE-BSA by macrophages. Under the identical conditions, the uptake of $^{125}$I-f-Alb by macrophages was also effectively inhibited by these AGE-proteins. These results suggest that the structure in common among these AGE-proteins might play an important role in the ligand recognition by the receptor. However, it remains unknown whether the structure involving this recognition might stem from the late-stage Maillard reaction or from the early-stage compounds such as a Schiff base adduct and Amadori rearrangement products.

To test this, we used reducing agents such as NaCNBH$_3$ and NaBH$_4$. It is known that a labile Schiff base adduct formed between glucose and protein amino groups is reduced to glucitollysine with NaCNBH$_3$ or NaBH$_4$, whereas only NaBH$_4$ is effective in reduction of Amadori rearrangement products to glucitollysine (26-28). When incubation of BSA with glucose was started in the presence of NaCNBH$_3$ or NaBH$_4$, no ligand activity was generated from any of these preparations (Table I). However, when AGE-BSA with the full ligand activity was treated with NaCNBH$_3$ or NaBH$_4$ for 4 h at 37°C, no change in the ligand activity occurred with AGE-BSA (Table I). These results suggest that the structure recognized by the receptor is generated through a series of the early-stage Maillard reaction. However, these early-stage products themselves, such as Schiff base adduct or Amadori rearrangement compounds, do not serve as a recognition signal. This notion was supported by the similar experiment with the receptor for aldehyde-modified protein. BSA preparations which had been treated with glycolaldehyde in the presence of NaBH$_4$ or NaCNBH$_3$ failed to compete with $^{125}$I-f-Alb for its cellular binding, while once the ligand activity was generated, the treatment with NaBH$_4$ or NaCNBH$_3$ was no longer effective for the ligand activity of glycol-Alb (Table I).

![Figure 9. Cross-competitive effect of f-Alb and AGE-BSA on their hepatic uptake. A, rats were injected intravenously with 8.3 μg of $^{125}$I-AGE-BSA alone or 3 mg of unlabeled AGE-BSA or f-Alb. After 10 min, the livers were flushed with ice-cold buffer, excised, and homogenized to determine the hepatic uptake as described under “Materials and Methods.” B, $^{125}$I-f-Alb (9.6 μg) was injected into each rat. Effects of unlabeled ligands were determined as described for A.](image-url)

**Table I**

<table>
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<th>Effector</th>
<th>Uptake by macrophages</th>
<th>Uptake by sinusoidal cells</th>
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<tr>
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<td>101</td>
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<td>BSA + glucose + NaCNBH$_3$ (NaBH$_4$)</td>
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<tr>
<td>Glycol-Alb + NaCNBH$_3$ (NaBH$_4$)</td>
<td>26 (28)</td>
<td>7 (6)</td>
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hemoglobin and AGE-human serum albumin equally well; BSA prepared very similar to each other; well known posttranslational modifications that occurs read-to  this issue. The nonenzymatic glycosylation is one of the
tween f-Alb and AGE-BSA in

The in vivo presence of the scavenger receptor system for f-Alb (24, 25) and other aldehyde-modified proteins (10) was demonstrated. However, its physiological significance had remained unknown since chemical modification of proteins by formaldehyde and other aldehydes is considered unlikely to occur in vivo to circulating proteins. Identification of a natural ligand for this receptor had indeed been a major clue to this issue. The nonenzymatic glycosylation is one of the well known posttranslational modifications that occurs readily in vivo (6, 29). Hence, it is reasonable to assume that AGE-proteins generated by the Maillard reaction should be a potential candidate for natural ligands of the scavenger receptor for aldehyde-modified proteins. This contention is supported by three lines of evidence: (i) cross-competition assays between f-Alb and AGE-BSA in in vitro binding, endocytic uptake, in vivo plasma clearance, as well as hepatic uptake demonstrated that receptors for these ligands are identical or very similar to each other; (ii) glucose is one of the physiological aldehydes, and the mode of chemical reaction leading to formation of AGE-BSA that exhibits the full ligand activity is very similar to that of f-Alb and glycol-Alb (Table I); and (iii) a receptor for AGE-BSA recognized both AGE-human hemoglobin and AGE-human serum albumin equally well (Table I), a mode of ligand recognition similar to that by the scavenger receptor for aldehyde-modified proteins.

Biological recognition of AGE-proteins by macrophages was discovered by Vlassara et al (14, 30). Myelin, a major nerve capsule protein, underwent nonenzymic glycosylation in vivo under hyperglycemia as well as in vitro to form AGE-myelin, which was endocytosed by mouse peritoneal macrophages in a receptor-mediated fashion (30). This was extended to BSA: mouse peritoneal macrophages avidly took up AGE-BSA prepared in vitro (14). The present study confirms their observation and adds a novel view that AGE-proteins might serve as ligands of the scavenger receptor for aldehyde-modified proteins. The physiological role of the scavenger receptor for AGE-proteins is not clear. When these AGE-proteins are produced in intravascular space or released into the blood stream, they would be avidly taken up by sinusoidal liver cells. When produced in situ or in an extravascular space, AGE-proteins might also be recognized by migrating macrophages. It is not clear in this case, however, whether the biological system may act in the elimination from tissues of AGE-proteins that are otherwise harmful to living organisms, or whether it may help accelerate the accumulation of the modified proteins in situ, which could eventually serve as a causative factor for diabetic complications or aging processes. A similar possibility was suggested for the receptor-mediated recognition of AGE-myelin by macrophages and its relation to the pathogenesis of segmental demyelination in diabetic neuropathy (30, 31). Further studies of the functional involvement of the scavenger receptor for aldehyde-modified proteins in these pathological processes using diabetic animals may shed some light on this issue.

Incubation of protein amino groups with glucose leads to a stable Amadori product via a Schiff base adduct. Upon further incubation this early stage reaction proceeds to the advanced stage with generation of products characterized by brown pigment, fluorescence, and protein-protein cross-linking (15). The in vivo presence of early stage products particularly in diabetic patients is known to several proteins with a relatively short half-life such as serum albumin, low density lipoprotein, high density lipoprotein (32–35), and hemoglobin (28). Although the functional alteration by early stage modification occurs to some of these proteins, it is generally believed that the late stage modification of long-lived proteins might contribute to a greater extent to pathogenesis of the diabetic complications or aging process (36–38). For instance, AGE-products by late stage Maillard reaction were demonstrated to occur in vivo in long-lived proteins such as collagen or lens crystallins, and AGE-induced functional impairments of these proteins were studied from their potential link to diabetic complications. However, it remains unknown whether the receptor-mediated recognition of AGE-proteins has some functional role in the pathogenesis.

In contrast to the well defined mechanism for the early stage reaction, little was known about the advanced stage Maillard reaction until a recent discovery of FFI as a major fluorescent product of AGE-proteins (16). Its in vitro and in situ presence was suggested by the immunological assay (17). In addition, it was also revealed that the FFI structure serves as an important signal for the receptor-mediated recognition of AGE-BSA by mouse peritoneal macrophages (14). Although the presence of a recognition system for AGE-proteins in macrophages was confirmed, the results of the present study argue against the potential involvement of FFI in this biological system: (i) FFI and its derivatives had no effect on the binding of 125I-AGE-BSA to macrophages or sinusoidal cells (Fig. 3) and on its plasma clearance rate (Fig. 4A); (ii) 125I-FFI-BSA did not exhibit any specific binding to these cells (Fig. 5), and its plasma clearance rate was comparable to that for unmodified BSA (Fig. 4B). These observations conform to the result of a preliminary experiment in which the radioimmunoassay using anti-FFI antibody failed to detect FFI in AGE-BSA. Furthermore, acid hydrolysis and subsequent reaction with ammonia were found to be essential for the generation of FFI from AGE-proteins, suggesting the absence of FFI in AGE-proteins.2 Thus, taken together, it is likely that a chemical structure other than FFI might be responsible for the receptor-mediated recognition of AGE-proteins.

Correlation of the chemical features of ligands with their activity was very similar between AGE-BSA and aldehyde-modified proteins such as f-Alb and glycol-Alb. First, once generated, the ligand activity of AGE-BSA was stable to reduction by NaBH4 or NaCNBH3 (Table I) and to acid or alkaline treatment, or to treatment with denaturants such as 8 M urea and 6 M guanidine hydrochloride,2 results identical to that of f-Alb (7). Second, the modification of lysine residues is essential in both cases. In fact, amino acid analysis of AGE-BSA used in the present study revealed a significant loss of lysine residues (~50%). On the other hand, some differences also exist between these ligands. The Kd values for AGE-BSA

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1) Previous experiments on the effect of these reducing agents on the generation of the ligand activity for f-Alb led to the similar result (10, 11). In view of the apparently common route leading to generation of a full ligand activity between AGE-proteins and aldehyde-modified proteins, the identity or close similarity between a receptor for AGE-proteins and that for aldehyde-modified proteins was also suggested.

and f-Alb in binding to sinusoidal liver cells as well as to peritoneal macrophages are of the same order of magnitude, whereas the maximal ligand binding capacity of AGE-BSA was about 3 times higher than that of f-Alb in sinusoidal liver cells (Fig. 7) and >8 times higher in peritoneal macrophages (Fig. 6 and Table 1). The inhibitory effect of f-Alb on the endocytic uptake of ¹²⁵I-AGE-BSA was weaker than that of unlabeled AGE-BSA (Figs. 6A and 7A). These findings do not exclude the possibility that AGE-BSA and aldehyde-modified proteins might be recognized by two different receptors but are very similar to each other. Further comparison will be needed between the purified receptor for AGE-proteins and the scavenger receptor for aldehyde-modified proteins.

REFERENCES