Two Novel Rat Liver Membrane Proteins that Bind Advanced Glycosylation Endproducts: Relationship to Macrophage Receptor for Glucose-modified Proteins

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Summary

Advanced glycosylation endproducts (AGEs), the glucose-derived adducts that form nonenzymatically and accumulate on tissue proteins, are implicated in many chronic complications associated with diabetes and aging. We have previously described a monocyte/macrophage surface receptor system thought to coordinate AGE protein removal and tissue remodeling, and purified a corresponding 90-kD AGE-binding protein from the murine RAW 264.7 cell line. To identify AGE-binding proteins in normal animals, the tissue distribution of ^{125}I-AGE rat serum albumin taken up from the blood was determined in rats in vivo. These uptake studies demonstrated that the liver was a major site of AGE protein sequestration. Using a solid-phase assay system involving the immobilization of solubilized membrane proteins onto nitrocellulose to monitor binding activity, and several purification steps including affinity chromatography over an AGE bovine serum albumin matrix, two rat liver membrane proteins were isolated that specifically bound AGEs, one migrating at 60 kD (p60) and the other at 90 kD (p90) on SDS-PAGE. NH_2-terminal sequence analysis revealed no significant homology between these two proteins nor to any molecules available in sequence databases. Flow cytometric analyses using avian antibodies to purified rat p60 and p90 demonstrated that both proteins are present on rat monocytes and macrophages. Competition studies revealed no crossreactivity between the two antisera; anti-p60 and anti-p90 antisera prevented AGE-protein binding to rat macrophages when added alone or in combination. These results indicate that rat liver contains at least two novel and distinct proteins that recognize AGE-modified macromolecules, although p90 may be related to the previously described 90-kD AGE receptor isolated from RAW 264.7 cells. The constitutive expression of AGE-binding proteins on rat monocytes and macrophages, and the sequestration of circulating AGE-modified proteins by the liver, provides further evidence in support of a role for these molecules in the normal removal of proteins marked as senescent by accumulated glucose-derived covalent addition products, or AGEs.

Glucose and other reducing sugars attach nonenzymatically to the amino groups of proteins in a concentration-dependent manner. Over time, these initial Amadori adducts undergo further rearrangements, dehydrations, and cross-linking with other proteins to accumulate as a family of complex structures that are referred to as advanced glycosylation endproducts (AGEs)[1]. Although this chemistry has been studied by food chemists for many years, it was only in the past decade that the presence of AGEs in living tissues has been established. The excessive deposition of these products on structural body proteins as a function of age and elevated glucose concentration, (1) taken together with evidence of effective prevention of tissue pathology by an AGE inhibitor, aminoguanidine (2), has lent support to the hypothesis that the formation of AGEs plays a role in the long term complications of aging and diabetes.

Since the amount of AGEs found in human tissues is less than could be predicted from protein/glucose incubation studies in vitro, we proposed several years ago that there might be normal mechanisms to remove those long-lived proteins that had accumulated AGEs in vivo. In fact, monocytes/mac-

[1] *Abbreviations used in this paper:* AGE, advanced glycosylation endproduct; FFI, 2-furoyl-4-(5)-(2-furanyl)-1H-imidazole; NC, nitrocellulose; RSA, rat serum albumin; TBIR, tissue-to-blood isotope ratio.

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rophages were found to display high affinity surface binding activity specific for AGE moieties, independent of the protein that was AGE modified. This macrophage AGE receptor was shown to differ from other known scavenger receptors on these cells (3, 4). The uptake and degradation of senescent, AGE-modified proteins by macrophages was also shown to be associated with the release of potent, multifunctional cytokines (IL-1 and TNF [5]) and growth factors (PDGF [6] and IGF-1[7]), suggesting that the AGE receptor system plays an important role in normal tissue remodeling. Recently, we have isolated a 90-kD protein from the membranes of the mouse macrophage cell line, RAW 264.7, which binds specifically to AGE proteins as well as to a chemically prepared model AGE compound, 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole, or FFI (8, 9).

Several other cell types, e.g., human endothelial (10) and mesangial cells (11), as well as human fibroblasts (12) also express membrane proteins that bind AGE-modified proteins; however, none of these recognize the model AGE compound, FFI. The exposure of endothelial cells in vitro to AGE proteins promotes procoagulant activity, associated with decreased membrane-associated thrombomodulin and increased production of tissue factor, as well as disruption of the barrier integrity of cultured endothelial monolayers (10). The interaction of mesangial cells or fibroblasts with AGE protein appears to stimulate increased matrix protein synthesis (11) and altered mitogenic activity (11, 12). Given the different binding specificities and functional characteristics of the AGE receptors present on these cell types compared to the receptor identified on macrophages, it is not clear whether these molecules are interrelated.

In the present paper, we report the purification from rat liver, and partial amino acid sequences, of two membrane proteins of ~60 and ~90 kD, respectively, that bind AGE-modified proteins. Both of these proteins are expressed on the surface of rat monocytes and resident peritoneal macrophages, suggesting a relationship to the AGE receptor system earlier identified on these cells and believed to be involved in tissue repair and remodelling.

Materials and Methods

Chemicals and Reagents. BSA (fraction V), bovine ribonuclease, glucosamide-BSA, glucose-6-phosphate, and collagen I were purchased from Sigma Chemical Co. (St. Louis, MO). Triton X-114 and Triton X-100 were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Sodium ^{125}iodide was obtained from New England Nuclear (Boston, MA). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). CNBr-activated Sepharose 4B was purchased from Pierce Chemical Co. (Rockford, IL). LDL and acetyl-LDL were the generous gift of Dr. David Via (Baylor College of Medicine, Houston, TX). 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI)[1] and its derivative, FFI-hexanoic acid (FFI-HA) were kindly provided by Dr. Peter Ulrich (Geritech Inc., Northvale, NJ).

Preparation of AGE Proteins and Formaldehyde-modified Proteins. AGE-BSA, AGE rat serum albumin (RSA), AGE ribonuclease, and AGE collagen I were generated by standardized methods as previously described in detail (3). In brief, each protein solution (25 mg/ml) was incubated with either 0.5 M glucose or glucose-6-phosphate in 100 mM phosphate buffer (pH 7.4) at 37°C for 6 wk under sterile conditions, and then low molecular weight reactants were removed by dialysis against PBS (20 mM sodium phosphate buffer containing 0.15 M NaCl, pH 7.4). FFI-HA was coupled to BSA with the water-soluble carbodiimide, EDAC, as described previously (13). Characteristic fluorescence of the AGE proteins was observed at 450 nm upon excitation at 390 nm (14). Formaldehyde-modified BSA was prepared as described (15), by incubating BSA in 0.1 M sodium carbonate buffer (pH 10) with 0.33 M formaldehyde at 37°C for 5 h, followed by extensive dialysis against PBS. All protein concentrations were determined by the method of Bradford (16). Protein and AGE protein preparations were radio-labeled with ^{125}I by the Iodo-Gen method (17). 2 mg of AGE-BSA was incubated with 25 mCi carrier-free ^{125}I in an Iodogen-coated glass vial at room temperature for 45 min. To separate free from bound ^{125}I, the sample was fractionated by Sephadex G-25M column chromatography and dialysis against PBS, until at least 98% of the ^{125}I was TCA precipitable. The specific radioactivity of the labeled ^{125}I-AGE-BSA was between 8,000 and 15,000 cpm/ng protein.

BSA and AGE-BSA Sepharose were prepared by reacting either BSA or AGE-BSA (25 mg/ml) with CNBr-Sepharose gel (5 ml/g of dry powder) according to the manufacturers instructions, in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). The mixture was rotated for 2 h at room temperature. Excess ligand was washed from the resin with coupling buffer and then with Tris-HCl buffer (0.1 M, pH 8.0) for 2 h at room temperature to block remaining active groups. The resin was then washed with three cycles of sodium acetate buffer (0.1 M, pH 4) containing NaCl (0.5 M), followed by Tris-HCl buffer (0.1 M, pH 8) containing NaCl (0.5 M), and stored at 4-8°C.

In Vivo Studies. For tissue distribution studies, AGE-RSA was prepared as described above (4), and radioiodinated to a specific activity of 8.3×10^5 cpm/μg. Similarly, normal RSA was iodinated to a specific activity of 6.2×10^5 cpm/μg. Freshly drawn rat RBC were labeled with ^{51}Cr to allow subsequent correction for tissue counts for blood-associated radioactivity. Approximately 1 mCi of ^{51}Cr was added to 10 ml RBC, mixed thoroughly, and allowed to incubate for 30 min at room temperature before washing with PBS containing 1% BSA and 0.1% dextrose until supernatant radioactivity was <1% of that in the packed RBC (18). Buffer was added to labeled RBC to obtain a hematocrit of 40% and the labeled RBC were used immediately.

To determine the tissue distribution of AGE ligands, normal male Sprague-Dawley rats (200 g) were divided into groups of five, and anesthetized with sodium pentobarbital (40 mg/kg). All animals received an injection of ^{51}Cr-RBC (0.65 ml, i.v.) 5 min before the labeled ligand. The indicated groups of rats received either ^{125}I-AGE-RSA (50 μg in 0.1 ml, i.v.) or an identical amount of ^{125}I-normal RSA. At the indicated time intervals, 0.5-ml aliquots of blood were drawn and various organs were removed and counted for radioactivity (19). The specificity of AGE ligand uptake in various organs was assessed by injecting groups of animals with excess nonlabeled AGE-BSA (5 mg) 2 min before administration of 50 μg of ^{125}I-AGE-BSA. After 5, 20, and 60 min, 0.5-ml blood samples were collected, animals were killed, and various organs and tissues were collected and counted for radioactivity. The RBC were lysed with water, and protein was precipitated with 20% TCA. The organs were weighed, homogenized with a hand homogenizer, protein was precipitated with 20% TCA, and counted for radioactivity. The tissue-to-blood isotope ratio (TBIR) was calculated by the formula: TBIR = (^{125}I/^{51}Cr in tissue)/(^{125}I/^{51}Cr in blood)

(20). TBIR is a dimensionless index of the degree to which any tissue sample has sequestered labeled ligand relative to the blood.

Solubilization and Fractionation of Hepatic Membrane Proteins. Liver membranes were prepared according to the method of Thom et al. (21), with some modifications. For a typical membrane preparation, 14 g of rat liver was homogenized in 80 ml TNE buffer (50 mM Tris-HCl buffer [pH 8.0], containing 150 mM NaCl, 0.1 mM EDTA, and 23 μg/ml PMSF and centrifuged for 10 min at 3,000 g. The supernatant was layered on top of a solution of 40% sucrose in TNE buffer, and centrifuged at 24,000 g for 1 h at 4°C. The membranes were collected from the interface with a Pasteur pipette. The membrane preparation was solubilized in TNE buffer containing 2% Triton X-114 at 4°C and clarified by centrifugation for 30 min at 100,000 g. The supernatant was then warmed to 30°C and the detergent phase, aqueous phase, and detergent-insoluble pellet were separated according to the phase separation method described by Bordier (22). The resulting detergent phase was either used directly for purification of AGE-binding proteins or diluted 1:10 with PBS containing 2% Triton X-100 and 2 mM PMSF. This material (D phase) was frozen at −80°C until further use.

In Vitro Solid-phase AGE-binding Assay. AGE-binding activity was determined by a modified version of solid-phase binding assay developed for the IL-1 receptor (23). Aliquots of detergent-solubilized membrane proteins were blotted onto grid-marked nitrocellulose (NC) membranes. The blots were dried at room temperature and could be stored at room temperature for several weeks without apparent loss of binding activity. The NC membranes were cut into small squares (0.9 cm^2) with the immobilized protein at the center and distributed in 24-well trays (Costar, Cambridge, MA). Immobilized protein was reconstituted in PBS, pH 7.4, containing 0.5% Triton X-100 for 30–60 min at room temperature. The blots were subsequently incubated in blocking buffer (PBS, pH 7.4, containing 2% BSA, 0.2% Triton X-100, and 1 mM $MgCl_2$) for 2 h with agitation at 4°C. Specific ligand binding was carried out by adding 50–100 nM ^{125}I-AGE-BSA directly to the blocking buffer and agitating at 4°C for a further 1.5 h. The NC membranes were transferred to a new tray and rinsed quickly three times with PBS containing 0.2% Triton X-100, followed by two additional 10-min washings with PBS. Ligand binding was then evaluated by autoradiography or gamma counting.

Ligand Blotting. SDS-PAGE and electro-blotting were performed as previously described (24). Proteins were electrophoretically separated on either 8–16% or 4–20% gradient polyacrylamide gels. After electro-blotting the proteins from the gel onto NC membranes, the blots were washed at 4°C overnight with PBS containing 0.2% Triton X-100. The blots were then incubated in blocking buffer for 3 h at 4°C with agitation. Ligand binding was performed by adding 10 nM ^{125}I-AGE-BSA to the blocking buffer. After 1.5–2 h at 4°C, the blots were washed three times with blocking buffer for 1 min each and two times for 10 min each. After air drying, the ligand binding was evaluated by autoradiography.

Isolation of p60 and p90 from Rat Liver Membranes. Unless otherwise indicated, all purification procedures were performed at 4°C. The detergent phase of the rat membrane preparation (D phase, ~860 mg protein) was applied to a polyethylenimine cellulose column (PEI) (3 × 30 cm) equilibrated with TNE buffer. After washing with equilibration buffer (TNE plus 2 mM PMSF containing 1% CHAPS), the proteins bound to the PEI column were eluted by a 240-ml linear gradient of 0.1–1.5 M NaCl in the equilibration buffer. Fractions were analyzed for binding activity by the solid-phase AGE binding assay. The active fractions were pooled and dialyzed overnight against TNE buffer containing 1% CHAPS. The PEI pool (160 mg protein, 80 ml) was then applied to a DEAE-cellulose column (2.5 × 20 cm) previously equilibrated with TNE/CHAPS/PMSF equilibration buffer. The column was washed with four-column volumes of equilibration buffer and proteins were eluted by a 200-ml linear gradient of 0.2-1.5 M NaCl in equilibration buffer. The fractions that contained AGE-BSA binding activity were pooled and concentrated by ultrafiltration (Centricon 10; Amicon). The concentrated DEAE pool (80 mg protein) was cycled three times through a BSA-Sepharose 4B column (2 × 12 cm, 10 mg of BSA per ml of gel), to eliminate proteins that bound to BSA. The flow-through from this BSA-Sepharose column was then applied to an AGE-BSA-Sepharose 4B column (2 × 6 cm, 10 mg of AGE-BSA per ml of gel) and cycled twice. The column was washed with 25-column volumes of PBS buffer, pH 7.4, containing 0.2% Triton X-100, and 1 mM PMSF. The proteins bound to the AGE-BSA column were eluted with the step-wise addition of PBS buffer containing 1.5 M NaCl, 0.2% Triton X-100, and 1 mM PMSF. Each fraction was dialyzed against PBS containing 1 mM PMSF, concentrated by ultrafiltration (Centricon 30, Amicon) and analyzed for ^{125}I-AGE-BSA binding activity by the solid-phase binding assay.

Preparative electrophoresis was performed as described in detail elsewhere (25). In brief, 50 μg of the protein mixture that had been affinity purified over AGE-BSA was boiled for 3 min in sample buffer (0.03 M Tris-HCl, pH 6.8, 1% SDS, 5% glycerol, 0.015% Bromophenol Blue) in the presence of 0.1 M 2-mercaptoethanol and subjected to electrophoresis through 10% polyacrylamide gels in the presence of 0.1% SDS. The 60-kD and 90-kD protein bands were excised and electro-eluted (Elutrap; Schleicher & Schuell) in Tris buffer (25 mM, pH 8.5) containing glycine (192 mM) and 0.1% SDS, as described (26).

Antibody Generation. Laying hens were injected subcutaneously at multiple sites with a total of 100–150 μg of electrophoretically purified p60 or p90 in CFA (Pocono Rabbit Farm and Laboratory, Canadensis, PA). On days 14 and 21, the hens were injected with an additional 60 μg of each protein in CFA. Further boosts of 80–100 μg of the corresponding proteins in IFA were given 1 mo after the initial series. Eggs and serum from the chickens immunized with p60 or p90 proteins were separately collected. Igs from the yolks were extracted according to the method of Polson et al. (27), while serum Igs were isolated by a combination of ammonium sulphate (30%) precipitation and DEAE cellulose chromatography (28).

Western Blotting. 10-μg aliquots of detergent-solubilized samples of membrane protein were boiled in sample buffer in the presence of 0.1 M 2-mercaptoethanol, and subjected to electrophoresis through gradient gels (8–16%). After transferring onto nitrocellulose, the membranes were rinsed in TBS-t buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween 20) and blocked with TBS-t buffer containing 2% BSA for 1 h at 4°C. The blots were probed with either anti-60 kD or anti-90 kD avian IgG or pre-immune chicken IgG (10 μg/ml) for 60 min at 4°C and then washed with TBS-t buffer three times, for 5 min each. The blots were then incubated with goat anti–chicken alkaline phosphatase conjugate (1:1,000 dilution) for 1 h at 4°C. Color development was achieved according to instructions of the manufacturer (Promega, Cleveland, OH).

Flow Cytometric Analysis: Cell Preparation. Heparinized blood was drawn from male Sprague-Dawley rats (200–300 g) by cardiac puncture. Purified monocytes were prepared over Ficoll-Hypaque (29) and Percoll gradients (30). Resident rat peritoneal macrophages were obtained from rats by washing the peritoneal cavity with 20 ml of PBS (4) and were characterized by flow cytometry.

Flow Cytometric Analysis: Fluorescence Flow Cytometry. The expression of p60 and p90 AGE-binding proteins on rat monocytes and macrophages was determined by indirect immunofluorescence. Single-color cell staining was performed by incubating 10^6 cells

with biotinylated anti-p60 or anti-p90 primary antibodies at a final concentration of 5 μg/ml for 20 min at 4°C. Cells were washed in staining buffer (PBS, 3% FBS, 0.1% NaN_3) and then incubated with FITC-conjugated avidin (Becton Dickinson & Co., Mountain View, CA). Background fluorescence was determined by staining the cells with a relevant isotypic control antibody, biotinylated chicken IgG used in identical concentrations (5 μg/ml). Cells were analyzed using a FACSCAN® (Becton Dickinson & Co.) with gates set by forward angle light scatter and side scatter. Fluorescence emission for FITC was detected by selectively collecting at 500–537 nm on at least 5,000 labeled cells, gated to include monocytes/macrophages and to exclude lymphocytes, other nonmonocytic cells and dead cells. The data were analyzed by Paint-A-Gate software (Consort 30; Becton Dickinson & Co.).

For the cross-competition study, 10^6 rat monocytes were treated with either 5 μg/ml biotinylated anti-p60 antibodies in the presence of 20-fold excess anti-p90 antibodies, or 5 μg/20 μl biotinylated anti-p90 in the presence of 20-fold excess anti-p60. The antibody-treated cells were then labeled using FITC-avidin and analyzed by flow cytometry.

Results

In Vivo Tissue Distribution of AGE-binding Activity. We have previously identified a 90-kD protein on mouse and human monocytes/macrophages which selectively binds AGE proteins (3, 4, 8, 9). Since these sources are not convenient to provide sufficient material for further biochemical characterization, we searched for alternate tissue sources. As a first step, the distribution of AGE-specific binding activity in rat tissues was examined by uptake studies of ^{125}I-AGE-RSA. Either ^{125}I-AGE-RSA (50 μg, 8.3 × 10^5 cpm/μg) or ^{125}I-normal RSA (50 μg, 6.2 × 10^5 cpm/μg) was injected intravenously into rats along with ^{51}Cr-labeled RBC, as described in Materials and Methods. After 10 min, >50% of the AGE-RSA was concentrated in the liver, whereas the liver uptake of nonmodified ^{125}I-RSA was consistently <10% of the AGE-RSA values (Fig. 1 *A*). Tissue accumulation of AGE-RSA was not affected by the prior injection of 100-fold excess nonlabeled RSA (5 mg, not shown). In contrast, pre-treatment of rats with excess nonlabeled AGE-RSA (5 mg) decreased the accumulation of AGE-RSA in the liver by ~45% after 10-, 20-, and 60-min intervals (Fig. 1 *B*). The uptake of AGE-RSA remained uniformly low in all other major organs, with or without the nonlabeled competitor. It was apparent that the liver had a high specific capacity to accumulate AGE protein and therefore represented a potentially rich source for the isolation of the AGE-binding proteins.

AGE-binding Assay. To facilitate the isolation of the AGE-binding proteins from liver, we developed a solid-phase assay system involving the immobilization of detergent-solubilized membrane proteins onto nitrocellulose and probing for ligand-specific binding activity with ^{125}I-AGE-BSA as described in Materials and Methods. Fig. 2 *A* shows the effect of increasing amounts of crude liver membrane proteins on AGE ligand binding. Total AGE-BSA binding increases in proportion to the amount of membrane proteins immobilized on the filters, whereas nonspecific binding was negligible. The AGE-binding kinetics of these membrane proteins after blotting onto nitrocellulose is shown in Fig. 2 *B*. When ~8 μg of hepatic membrane proteins immobilized onto NC filters were incubated with increasing amounts of ^{125}I-AGE-BSA, saturable binding was observed with a B_{max} of 0.22 pmol/8 μg of protein (Fig. 2 *C*). A dissociation constant (K_d of 4 × 10^{-8} M) was revealed by Scatchard analysis of the binding data (31).

The specificity of liver cell binding activity for AGE adducts on protein was determined in competition experiments testing ^{125}I-AGE-BSA against several different AGE protein competitors, as well as against ligands known to bind to other scavenger receptors (Fig. 2 *D*). The addition of 150-fold excess AGE-RNAse or AGE-collagen I completely inhibited the

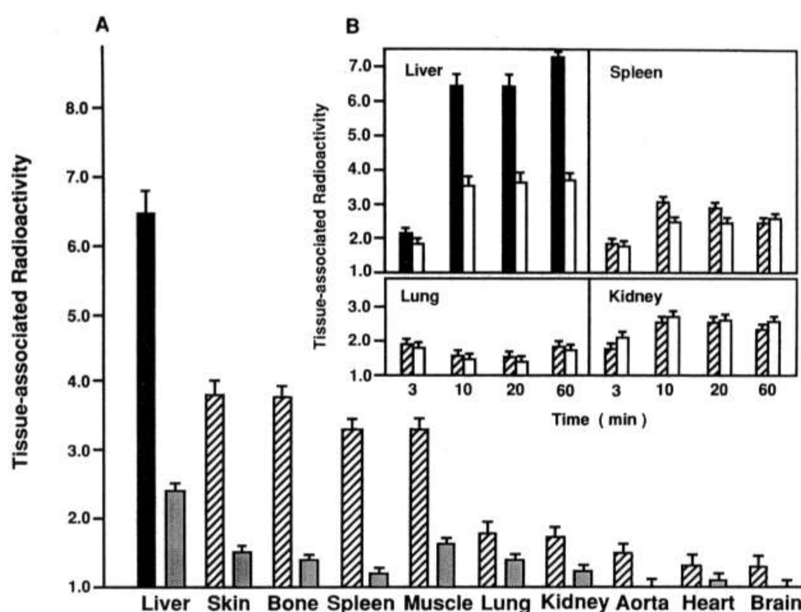

Figure 1. (*A*) In vivo tissue distribution of ^{125}I-AGE-RSA. 50 μg of either ^{125}I-AGE-RSA (*filled* and *striped bars*) or ^{125}I-native RSA (*shaded bars*) were administered intravenously to normal rats. At 10 min, the animals were killed, and the organs and tissues were removed and counted for radioactivity. Whole organ counts were corrected for blood-associated counts as described in Materials and Methods. (*B*) Specific competition of ^{125}I-AGE-RSA uptake in rat liver, spleen, lung, and kidney. Immediately before receiving radiolabeled ligand, rats were injected with excess nonlabeled AGE-RSA (5 mg, i.v.). At the indicated time intervals, incorporated radioactivity was determined in blood and tissues, and compared according to a TBIR formula as described in the text. (*Closed* and *striped bars*) ^{125}I-AGE-RSA (50 μg) alone; (*Open bars*) ^{125}I-AGE-RSA (50 μg) in the presence of excess nonlabeled AGE-RSA. Data are expressed as mean ± SEM of three independent measurements performed in five animals per group.

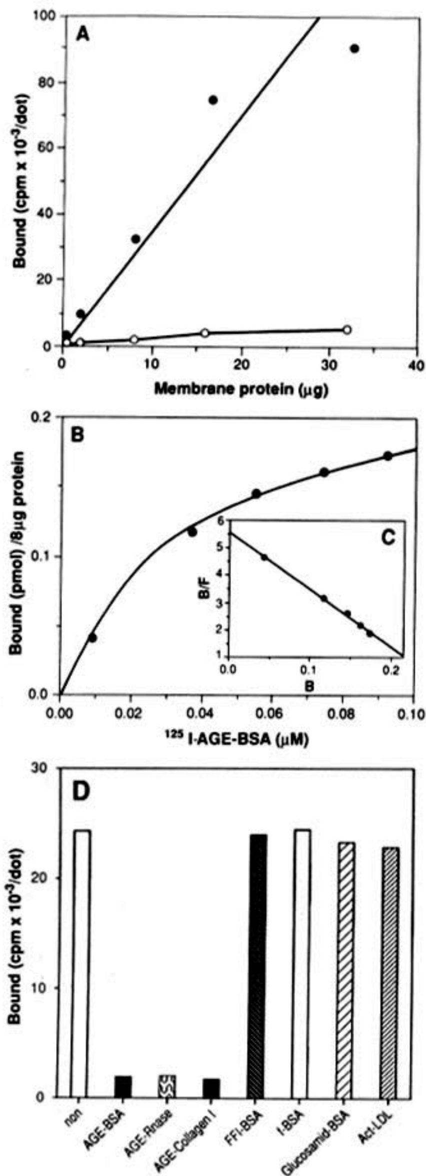

Figure 2. AGE-binding activity assay. (*A*) Relationship of liver membrane protein concentration to AGE-ligand binding. Aliquots (1–50 μg) of a detergent-solubilized liver membrane protein preparation were immobilized on nitrocellulose filters, incubated with blocking buffer, and then probed for AGE protein binding activity with ^{125}I-AGE-BSA in the presence or absence of excess nonlabeled AGE-BSA. After washing, the blots were counted for ^{125}I. (*Closed circles*) ^{125}I-AGE-BSA alone (total binding); (*open circles*) ^{125}I-AGE-BSA plus 100-fold excess nonlabeled AGE-BSA (nonspecific binding). Data points represent duplicate blots. (*B*) Saturability of ^{125}I-AGE-BSA binding. A fixed amount of solubilized liver membrane proteins (8 μg) was immobilized on duplicate nitrocellulose filters which were probed with increasing concentrations of ^{125}I-AGE-BSA (10–100 nM, s.a. 1.5 × 10^5 cpm/ng) in the presence (nonspecific) or absence (total binding) of 200-fold excess nonlabeled AGE-BSA. After washing, the nitrocellulose filters were counted for retained ^{125}I. Specific binding was determined by subtracting nonspecific binding from total binding. (*C*) Scatchard analysis of specific binding data (B = pmol/8 μg membrane protein, F = μM). (*D*) Effects of differently modified protein ligands on ^{125}I-AGE-BSA binding. Filter blots of solubilized liver membranes prepared as above were probed with ^{125}I-AGE-BSA (50 nM) alone, or in the presence of 150-fold excess of various nonlabeled competitors: AGE-BSA, AGE-RNAse, AGE-collagen I, FFI-BSA, formaldehyde-treated albumin (f-alb), glucosamide BSA, acetyl-LDL (act-LDL). Data are expressed as the amount of ^{125}I-AGE-BSA (cpm × 10^{-3}) retained on duplicate blots.

binding of radio-labeled AGE-BSA to crude hepatic membrane protein extracts immobilized on NC filters. In contrast FFI-BSA, formaldehyde-treated BSA, glucosamide-BSA (a chemically linked glucose-BSA compound) or acetyl-LDL did not compete against the binding of labeled AGE-BSA.

Using similar detergent-solubilized membrane preparations from heart, kidney, brain, or lung obtained by identical procedures as described for liver, ^{125}I-AGE-BSA-specific binding activity was examined by the same solid-phase AGE-binding assay. Liver membrane proteins exhibited the highest binding activity among all tissues examined, consistent with our in vivo observations (data not shown).

Purification of Rat Liver AGE-binding Proteins. Using the solid-phase AGE binding assay as a means of monitoring AGE-binding activity column fractions, we pursued the isolation of AGE-binding protein(s) by the procedure outlined in Table 1 and described in detail in Materials and Methods. In brief, rat liver membrane proteins were solubilized in Triton X-114. After detergent phase-separation, the D phase was subjected to chromatography on PEI-cellulose, DEAE-cellulose, BSA-Sepharose, and finally, AGE-BSA-Sepharose. After elution from the AGE-BSA Sepharose column, the fractions were assessed for AGE-binding activity by the solid-phase AGE binding method (Fig. 3). Analytical SDS-PAGE electrophoresis of the active fractions obtained from the AGE-BSA-column revealed the presence of two main protein bands with approximate molecular masses of 60 kD (p60) and 90 kD (p90) (Fig. 3, *inset*). To separate larger amounts of these AGE-binding proteins, AGE-BSA column eluate fractions were subjected to preparative PAGE and the individual proteins were separately electro-eluted from respective gel slices.

Gel-purified p60 and p90 were blotted onto Immobilon membranes and NH_2-terminal sequences were obtained at The Rockefeller University sequencing facility. Table 2 records the NH_2-terminal sequence obtained from each of these proteins. Comparison of these sequences with the translated Genbank database did not reveal significant similarity to other known proteins.

Ligand blotting of p60 and p90 proteins immobilized on nitrocellulose, using ^{125}I-AGE-BSA as probe, revealed that only the 60-kD protein bound this ligand (not shown). After blotting on nitrocellulose, the 90-kD protein did not bind ^{125}I-AGE-BSA, although p90 did bind to the AGE-BSA-Sepharose matrix and was not retained on the BSA-Sepharose column.

When crude rat liver membrane proteins (D phase) were separated by SDS-PAGE under nonreducing conditions, transferred to NC filters, and probed for ligand binding with ^{125}I-AGE-BSA, a single major AGE-BSA binding band at an approximate molecular mass of 60 kD was revealed (Fig. 4 *A*, lane *1*). The binding of this protein to AGE-BSA was inhibited partially in the presence of a 25-fold excess (lane *2*) and completely by a 150-fold excess of nonradioactive AGE-BSA (lane *3*). No other prominent bands were observed under

Table 1. *Purification of Rat Liver AGE-binding proteins*

Purification Step	Total Protein	^{125}I-AGE-BSA binding Total	Specific activity	Purification factor	Recovery
	mg	*µg*	*µg/mg*		%
Liver membrane	6,800	455.5	0.067	1	100
D-phase	830	207.5	0.256	3.7	45
PEI	175	262.2	1.498	22	58
DEAE	82	153.5	7.490	109	34
AGE-affinity	0.560	92.4	165	2463	20
Preparative PAGE*	0.180	–	–	–	–

Rat liver membrane proteins were procured and prepared as described. Specific binding of AGE-BSA was determined by the solid phase binding assay.
*Binding activity could not be determined due to the presence of SDS.

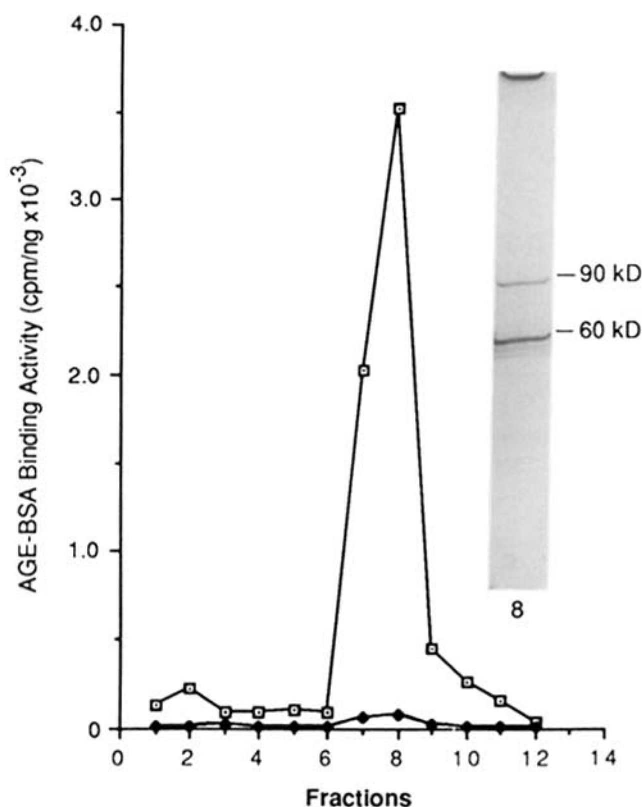

Figure 3. Purification of rat liver AGE-binding proteins. Detergent-solubilized membrane proteins were fractionated by successive PEI-cellulose, DEAE-cellulose, and BSA-Sepharose 4B column chromatography, as described in Materials and Methods. The flow-through from the BSA-Sepharose column was then applied to an AGE-BSA-Sepharose 4B column. This column was washed and bound proteins were eluted by the addition of high salt buffer. Each fraction was concentrated and analyzed for AGE-binding activity using the binding assay described in Fig. 2. (*Open circles*) Total binding activity; (*closed circles*) nonspecific activity. (*Inset*) SDS-PAGE analysis of fraction no. 8 (mercaptoethanol reduced), and stained with Coomassie blue.

these conditions. It thus appears likely that nitrocellulose immobilization may inactivate the binding properties of p90, or that p90 is a p60-associated protein that lacks independent AGE-binding activity.

Immuno-characterization of AGE-binding Proteins. Purified p60 and p90 proteins were injected into chickens in order to obtain specific polyclonal antibodies. Preparation of avian IgG specific for each of the proteins were isolated either from egg yolk or serum as described in Materials and Methods. The specificity of each of the antibodies was verified by Western blot analysis, using the same crude liver membrane protein extract analyzed above by ligand blotting (Fig. 4 *B*). The antibody to the p60 AGE-binding protein recognized a major protein band at ~60 kD (lane *2*), while the pre-immune IgG did not (lane *1*). Similarly, the antibody to p90 recognized a single protein band at ~90 kD (lane *4*), whereas the pre-immune antibodies did not (lane *3*).

The antibodies to p60 and p90 were used to screen for the expression of these proteins on the surface of rat peripheral blood monocytes and peritoneal macrophages. FACS® analyses, which demonstrated the presence of each protein on the surface of both cell types, are shown in Fig. 5. Fig. 5, *A* and *B*, illustrate flow cytometric detection of p60 and p90 on the surface of rat monocytes. Fig. 5 also shows that the binding of either anti-p60 or anti-p90 to the monocyte cell

Table 2. *NH$_2$-terminal Amino Acid Sequence Analysis of AGE-binding Proteins from Rat Liver*

Size	Sequence
60 kD:	XGPRTLVLLDNLNVRDTHXLFF
90 kD:	XEVKLPDMVSLXD

X indicates unidentified residues.

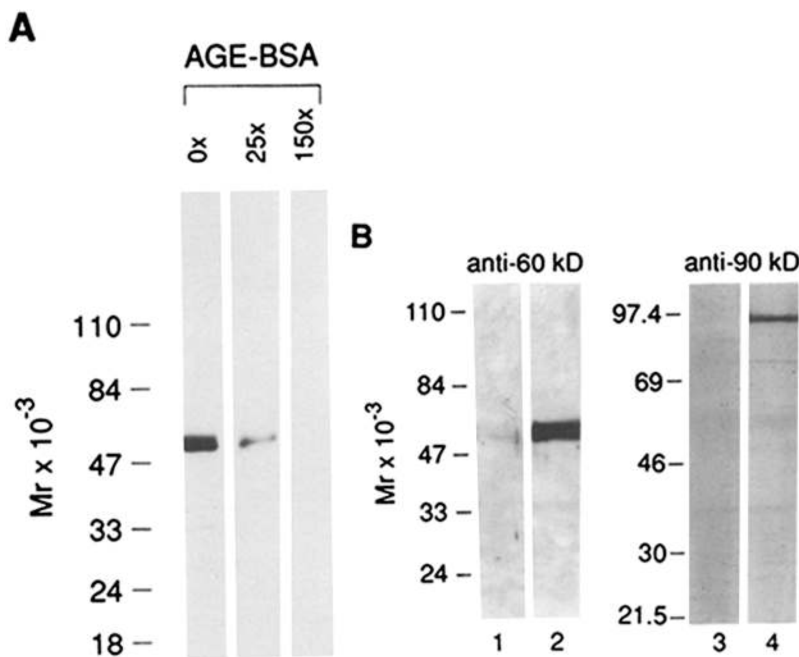

Figure 4. (*A*) Ligand blot analysis of rat liver membrane proteins. Aliquots (15 μg each) of detergent-solubilized membrane proteins were subjected to electrophoresis through 8–16% acrylamide gradient gel under nonreducing conditions and electro-transferred onto nitrocellulose filters. Using ^{125}I-AGE-BSA (50 nM, 8.0 × 10^5 cpm/ng) as probe, specific binding was determined in the presence of 0-, 25-, or 150-fold excess nonlabeled AGE-BSA using autoradiographic detection. Migration of molecular mass standards is shown at left. (*B*) Aliquots of solubilized membrane proteins (mercaptoethanol reduced), were subjected to electrophoresis through an 8–16% gradient gel and electro-transferred onto nitrocellulose filters. After washing and blocking with excess BSA, the blots were probed with purified IgG fractions of either anti-p60 or anti-p90 chicken antisera or the corresponding preimmune sera, and then exposed to goat anti–chicken antibody conjugated to alkaline phosphatase and reacted for phosphatase-dependent color development. Lane *1*, preimmune IgG; lane *2*, anti-p60 IgG; lane *3*, preimmune IgG, lane *4*, anti-p90 IgG. Results are representative of three independent experiments.

surface was not affected by a 20-fold excess of the heterologously directed antibody (*C* and *D*, respectively). Distinct binding of anti-p60 and anti-p90 antibodies was also observed when the rat peritoneal resident macrophages were analyzed by flow cytometry (Fig. 5, *E* and *F*, respectively). A small subgroup of highly fluorescent cells of an unspecified nature with a nonspecific FITC staining pattern was also noted, using antibody as well as isotypic controls.

To confirm that p60 and p90 were both AGE-binding proteins expressed independently on rat peritoneal resident macrophages, ^{125}I-AGE-BSA binding inhibition experiments were carried out using each antibody separately (undiluted: 10 μg/ml) as well as in combination (Fig. 6 *A*). In the presence of increasing concentrations of anti-p60 antibody, significant AGE-BSA binding inhibition was observed (up to 80% at a final concentration of 10 μg/ml). Similarly ^{125}I-AGE-BSA binding was inhibited up to 60% by the anti-p90 antibody, while the combination of both antibodies at a dilution of 1:10 provided 84% inhibition. When radiolabeled FFI-BSA (made from the chemically synthesized model AGE compound, FFI) (13) was used as the ligand, anti-p60 as well as anti-p90 mediated a concentration-dependent inhibition of FFI-modified BSA binding (Fig. 6 *B*). Moreover, and as found with AGE-BSA, a combination of anti-p60 and anti-p90 antibodies exerted greater inhibition compared to either anti-p60 alone or anti-p90 alone. No inhibitory effect was noted when isotypic control antibodies were used, even at the maximal concentration, in conjunction with either modified BSA ligand (data not shown).

Discussion

In the present communication, we describe the isolation of two novel rat liver membrane proteins, designated p60 and p90 by their migration in SDS-PAGE, which specifically bind to protein ligands modified by AGEs. NH_2-terminal sequence analysis indicates that these proteins bear no significant homology to each other nor to any previously sequenced proteins currently available in the Genbank database. Both p60 and p90 are present on rat monocytes and macrophages and are immunoreactively and functionally distinct. Of importance is the fact that these binding proteins have been distinguished from both the recently reported macrophage scavenger receptor for acetyl-LDL, a functional trimer composed of three 77-kD glycoprotein subunits (32), and from the binding proteins for formaldehyde-treated albumin with M_rs of 30 and 52 kD (33).

The p60 and p90 AGE-binding proteins were isolated from rat liver, once we had determined that this organ acts as a major filter for the in vivo clearance of AGE-modified macromolecules; liver presents the highest capacity to specifically sequester AGE proteins administered intravenously (Fig. 1). An isolation procedure, including elution of detergent-solubilized membrane proteins from an AGE-protein affinity matrix, was devised, and the p60 and p90 AGE-binding proteins were found to copurify. When immobilized on nitrocellulose, however, only p60 retained binding activity for AGE-modified ligands. Just as they copurified over anion exchange and ligand affinity columns, p60 and p90 were also observed to co-purify in hydroxylapatite chromatography (data not shown). Further experimentation is required to determine whether this copurification is fortuitous, or due to molecular interaction between the 60- and 90-kD proteins.

The liver is a complex organ containing several cell types, including macrophages and endothelial cells, both of which have been shown to bear AGE-receptors (3–6, 10, 30). It is alternatively possible that the distinct AGE-binding proteins we isolated from whole liver originated from different populations of cells, or that each protein was expressed with different

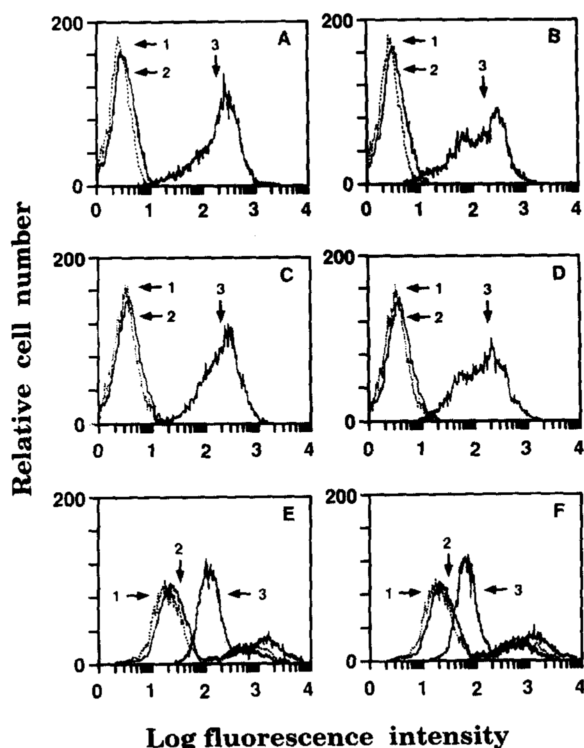

Figure 5. Demonstration by flow cytometry of expression of p60 and p90 AGE-binding proteins on rat monocytes and macrophages. Peripheral blood monocytes (*A–D*) and peritoneal resident macrophages (*E* and *F*) were treated with biotinylated anti-p60 alone (*A* and *E*), biotinylated anti-p90 alone (*B* and *F*), biotinylated anti-p60 + 20-fold excess unconjugated anti-p90 (*C*), biotinylated anti-p90 + 20-fold excess unconjugated anti-p60 (*D*), followed by FITC-avidin and analyzed by FACSCAN®. (Arrow *1*) Fluorescence of cells treated with FITC-avidin in the absence of either anti-p60 or anti-p90 (*dotted line*). (Arrow *2*) Fluorescence of cells treated with FITC-avidin subsequent to treatment with biotinylated chicken IgG (isotypic control). (Arrow *3*) Fluorescence of cells treated with FITC-avidin subsequent to treatment of cells with biotinylated anti-p60 (*A*, *C*, and *E*) and anti-p90 (*B*, *D*, and *F*). Note the lack of competition between a 20-fold excess of unconjugated anti-p90 for the binding of anti-p60 to the monocytes (panel *A* vs. *C*), and the lack of competition between a 20-fold excess of unconjugated anti-p60 for the binding of anti-p90 to the monocyte cell surface (panel *B* vs. *D*). All antibodies were used at a final concentrations of 5 μg/ml, unless otherwise indicated.

affinity and/or at a different density in a cell-specific manner. A 90-kD protein that binds to AGE-BSA, as well as to the chemically synthesized AGE model compound, FFI, has been previously described on murine macrophages (4, 8, 9, 13). To determine whether macrophages also expressed a 60-kD AGE-binding protein, and whether there was any relationship between the macrophage 90-kD and the liver p90 AGE-binding proteins, specific polyclonal antibodies to liver p60 and p90 were developed.

The specificity of these antibodies was demonstrated by Western analysis of crude liver membrane extracts, revealing that each antiserum identified a single protein band of the appropriate molecular weight. Flow cytometric analysis of rat peripheral monocytes and peritoneal resident macrophages revealed that each antisera bound to the surface of both of these cell types. Crosscompetition studies performed on monocytes revealed no crossreactivity between the two antibodies. These data indicate that the p60 and p90 AGE-binding molecules originally isolated from whole liver preparations are each present on monocytes as well as macrophages (Fig. 5).

Flow cytometric binding inhibition experiments clearly demonstrated that p60 and p90, expressed on the surface of monocytes/macrophages, independently bound AGE-modified ligands. Interestingly, a combination of antibodies specific for p60 and p90 mediated greater inhibition of AGE-protein binding than did either antibody alone.

Either antiserum, used independently or in combination, prevented >90% of binding of FFI-BSA to rat macrophages. In the case of p60, this finding is surprising given that this binding protein does not bind FFI-BSA in a solid-phase ligand blotting assay. Possibly, the binding affinity of this receptor protein for the FFI moiety is lower than that for other AGE structures contained in the heterogeneous AGE-modified protein preparations. With regard to liver p90, the flow cytometry and FFI-binding inhibitory data indicate that this molecule may be closely related to the 90-kD protein isolated from murine RAW 264.7 cells (9). In fact, preliminary experiments using antibodies raised against rat liver p60 and p90 proteins to stain mouse RAW cells provided flow cytometric and binding inhibition results similar to those obtained with rat monocytes and macrophages, strongly supporting a structural similarity between the AGE-binding proteins of these two rodent species.

There are several ways that these binding results, taken in sum, might be interpreted: it is possible that the p60 and p90 proteins represent structurally distinct subunits of a single receptor complex, each of which binds AGE moieties with differential affinity. Alternatively, both proteins may be present in equimolar concentration on the cell surface, but only p60 binds AGEs, while p90, although necessary to stabilize AGE binding, is itself incapable of independently immobilizing AGE ligands. The ability of the anti-90 antiserum to block AGE-binding according to this second interpretation would reflect a perturbation of p60-mediated AGE protein binding activity via the interaction of antibody with p90, suggesting that p60 and p90 exist in close association.

The AGE-specific receptor system now includes a variety of tissues and cell types in addition to monocyte/macrophages for which receptor-mediated AGE-protein internalization and digestion was first described (3, 4, 30). Endothelial cells (10), mesangial cells (11), and fibroblasts (12) have since been shown to specifically bind AGE-modified proteins (8). In macrophages, AGE protein uptake is accompanied by the release of a variety of potent cytokines and growth factors (5–7), which may coordinate processes of normal tissue remodeling. The other cell types do not bind the model compound AGE, FFI, nor are they known to release cytokines and growth factors in response to AGE-ligand binding, but each cell type does display distinct functional responses. For example, endothelial cells exhibit enhanced surface procoagulant activity and permeability (10), mesangial cells display enhanced matrix protein synthesis (11), while human fibroblasts exhibit increased proliferation upon exposure to AGEs (12). Although

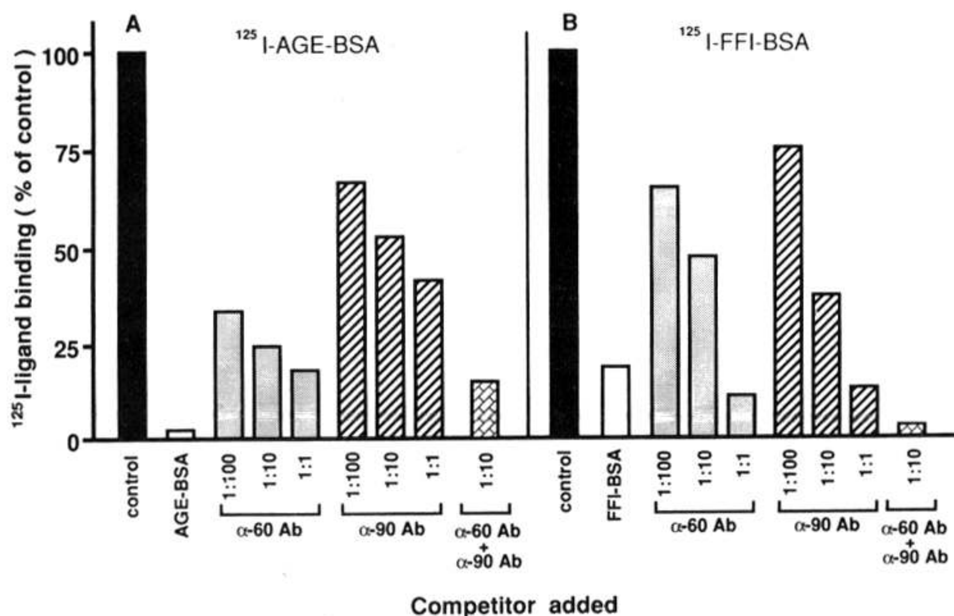

Figure 6. Inhibition of ^{125}I-AGE-BSA binding (*A*) and ^{125}I-FFI-BSA binding (*B*) on rat macrophage cell surface by anti-p60 and anti-p90 antibodies. Rat peritoneal resident macrophages were collected by peritoneal lavage and purified, then incubated with the indicated radiolabeled ligand in the presence or absence of a 10-fold excess of nonlabeled ligand or in the presence of antibodies to p60 or p90 at the indicated dilutions. Both antibodies were used alone (undiluted: 2 μg/200 μl) or in combination (at 1:10 dilution). Data are expressed as percent of maximal control binding (defined as the amount of ^{125}I-ligand bound to the cell surface in the presence of 10% FCS) and represent the mean of duplicate experiments.

our understanding of the receptor-mediated effects of AGEs is at an early stage, it is possible that there may be a family of AGE-binding proteins, distributed among different tissues, with distinct spectra of ligand specificities and inducible functions. In addition, given the broad heterogeneity of AGE moieties, one might anticipate the cooperation of more than one subunit, as might be reflected by the p60 and p90 proteins, in the AGE-uptake system of the macrophage. Additional studies are needed to define the specific recognition mechanisms that regulate the processing of senescent, AGE-modified proteins.

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