# Human and Rat Mesangial Cell Receptors for Glucose-modified Proteins: Potential Role in Kidney Tissue Remodelling and Diabetic Nephropathy

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## Summary

Advanced glycosylation endproducts (AGEs) are derived from the nonenzymatic addition of glucose to proteins. AGEs have been found to accumulate on tissue proteins in patients with diabetes, and their accumulation is thought to play a role in the development of diabetic complications. The finding that macrophages and endothelial cells contain AGE-specific receptors led us to examine whether mesangial cells (MCs) also possess a mechanism for recognizing and processing AGEs. Membrane extracts isolated from rat and human MCs were found to bind AGE–bovine serum albumin (BSA) in a saturable fashion, with a binding affinity of $2.0 \pm 0.4 \times 10^6$ $M^{-1}$ (500 nM). The binding was specific for the AGE adduct, since AGE-modified collagen I and ribonuclease both competitively inhibited $^{125}$I-AGE-BSA binding to MC membranes, while the unmodified proteins did not compete. Binding of AGE proteins was followed by slow internalization and degradation of the ligand. Ligand blotting of MC membrane extracts demonstrated three distinct AGE-binding membrane proteins of 50, 40, and 30 kD. Growth of MCs on various AGE-modified matrix proteins resulted in alterations in MC function, as demonstrated by enhanced production of fibronectin and decreased proliferation. These results point to the potential role that the interaction of AGE-modified proteins with MCs may play in vivo in promoting diabetic kidney disease.

In diabetes mellitus, expansion of the glomerular mesangium correlates with the clinical features of diabetic kidney disease, including albuminuria, hypertension, and decreased glomerular filtration rate (1–3). Mesangial expansion leads to a decrease in glomerular filtration rate by impinging on the glomerular capillary vasculature, thereby decreasing the filtering surface of the glomerulus. The increase in mesangial matrices is due primarily to the accumulation of normal matrix proteins, including collagens type IV and type V, laminin, and fibronectin (4, 5). While it is generally agreed that the altered physical milieu in diabetes is responsible for the development of diabetic kidney disease, the mechanisms by which hyperglycemia might lead to mesangial expansion are still poorly defined.

Experimental data have accumulated linking the formation of advanced glycosylation endproducts (AGEs)[1] to many of the complications of diabetes (6, 7). AGEs are derived from early products of nonenzymatic glycosylation, and are formed slowly from the early Amadori product after a series of reactions and rearrangements. AGEs, which represent irreversible late reacting products, are characterized by brown color, fluorescence, and their ability to cause protein-to-protein crosslinking (6, 7). AGE compounds have also been shown to bind to specific receptors on murine and human monocyte/macrophages, as well as bovine and human endothelial cells. Since AGEs form progressively as a function of time, it is hypothesized that under normal conditions, the function of AGE receptors is partly to signal cells such as the macrophage to promote turnover of aging tissue proteins and cells (8, 9). Under conditions of normoglycemia, the removal of AGE-modified proteins occurs conceivably at a rate sufficient to keep up with the production of new AGE proteins, preventing overt accumulation of AGE-modified proteins. However, in diabetes, excessive formation of AGEs in the presence of continuously elevated blood glucose may overwhelm the body's ability to remove AGEs, resulting in a net excess of AGEs on most structural tissue proteins (10). This hypothesis is supported by the demonstration that AGEs accumulate at an accelerated rate in diabetics on long-lived matrix proteins in the kidney and blood vessel wall (8, 11). Recent experimental evidence suggested that interaction of AGE-modified proteins with macrophages and endothelial

[1] *Abbreviations used in this paper:* AGE, advanced glycosylation endproduct; EC, endothelial cell; MC, mesangial cell.


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cells (ECs) can result not only in the binding and uptake of AGEs, but can also modulate cellular function (12, 13). Macrophages, upon interaction with AGEs, are induced to release the cytokines cachectin/TNF, IL-1, platelet-derived growth factor, and insulin-like growth factor I (12, 14, 15). Under conditions of normoglycemia, this response may be beneficial, aiding in the removal of AGE proteins and promoting normal tissue remodeling. However, excessive formation of AGEs in diabetics may lead to an exaggerated response, resulting in excessive production of these factors, all of which could contribute to diabetic complications such as premature development of atherosclerotic plaques or mesangial expansion in the kidney. More direct evidence supporting a role for AGE receptor interaction in the development of diabetic complications derives from experiments performed with cultured ECs (13). The interaction of ECs with AGEs induces several changes in EC function that are characteristic of diabetes, including an increase in EC permeability and an increase in EC procoagulant properties.

Since mesangial cells are primarily responsible for the maintenance of the glomerular mesangium (16, 17), we hypothesized that they may contain binding sites for AGE-modified proteins via which they may participate in the turnover of these proteins. In addition, we hypothesized that the accumulation of AGEs on mesangial matrix proteins in diabetes could directly modify mesangial cell (MC) function, resulting in altered proliferation and/or synthesis of matrix proteins. Our results indicate that MCs specifically bind AGE-modified proteins in a saturable fashion. In addition, MCs plated onto AGE-modified matrices demonstrate functional changes, including enhanced production of fibronectin and decreased proliferation. These results point to the potential role that the interaction of AGE-modified matrix proteins with native cells may play in vivo in promoting diabetic kidney disease.

## Materials and Methods

*Cell Culture.* Primary cultures of rat MCs were obtained from outgrowths of isolated rat glomeruli by Dr. M. Ganz (Yale University, New Haven, CT), as previously described (18, 19). In brief, rats were anesthetized with ether and the kidneys were excised under sterile conditions. After removing the kidney capsule, the kidney cortices were isolated, minced to a fine paste with a razor blade, and then pressed through serial stainless steel sieves (Nos. 140, 80, and 200; Fisher Scientific Co., Pittsburgh, PA). Glomeruli were collected from the top of the 75-$\mu$m sieve. This process resulted in >98% pure glomeruli. The glomeruli were then pelleted and resuspended in DMEM supplemented with 20% FCS, 5 $\mu$g/ml bovine insulin, 2 mM L-glutamine, and 40 $\mu$g/ml gentamicin. The glomerular suspensions were plated onto tissue culture flasks and incubated at 37°C in 5% $CO_2$. Primary cultures were allowed to grow for 3–4 wk, at which time the MCs were confluent. MCs were used between the fourth and ninth passages. The purity of the rat MC populations was documented by several criteria (18–20). The MCs exhibited a uniform straplike appearance and stained positively for Thy 1-1 antigen, myosin, and actin. They were sensitive to mitomycin C, a MC toxin, but were resistant to the aminonucleoside puromycin, an epithelial cell toxin. Fibroblast contamination was excluded by demonstrating the ability of the cells to grow in media in which L-valine had been substituted for D-valine. In addition, they stained negative for factor VIII and cytokeratin. Over the experimental period, they continued to maintain a uniform stellate appearance.

Human MC were provided by Dr. J. Floege and Dr. K. Resch (Hannover, FRG), prepared as previously described in detail (21). In brief, normal human kidney tissue was obtained from nephrectomy specimens. Renal cortices were homogenized and glomeruli were isolated after passage through a series of graded sieves. The glomeruli were then treated with bacterial collagenase (Worthington Biochemical Corporation, Freehold, NJ) at 37°C for 30 min, and after extensive washing, the glomerular remnants were plated onto tissue culture flasks in RPMI 1640, supplemented with 20% FCS, 2 mM L-glutamine, 2 mM sodium pyruvate, 5 $\mu$g/ml bovine insulin, 5 $\mu$g/ml human transferrin, 1% (vol/vol) nonessential amino acids, and gentamicin. Cellular outgrowths appeared between days 5 and 8, and all experiments were performed using cells between the fourth and tenth passage. The purity of the MC population was demonstrated as described in detail elsewhere (20). In brief, immunofluorescent staining demonstrated prominent intracellular staining for smooth muscle cell myosin, MHC class I antigen, vimentin, collagen IV, and fibronectin. The cells stained negative for Fc receptor, MHC II surface antigen, cytokeratin, and factor VIII, and were able to grow in D-valine-substituted medium.

*Preparation of Ligands.* AGE-BSA and AGE-ribonuclease were made by incubating BSA and bovine ribonuclease (Sigma Chemical Co., St. Louis, MO) with 0.5 M glucose-6-phosphate (G-6-P), at 37°C for 4–6 wk in a 10 mM PBS buffer, pH 7.4, in the presence of protease inhibitors and antibiotics as previously described (8). Unincorporated glucose was removed by dialysis against 1× PBS. The concentration of AGE-BSA was determined by the method of Bradford (22), and the concentration of ribonuclease was determined spectrophotometrically. AGE formed on either BSA or ribonuclease was assessed based on characteristic absorption and fluorescence spectra (emission at 450 nm, excitation at 390 nm) (23) and quantitated by a radioreceptor assay using intact RAW 264.7 cells grown in 96-well plates (11, 24). According to this assay, AGE-BSA contained ~70 AGE U/mg (1 U of AGE is defined as the concentration of unknown agent required to produce 50% inhibition of standard $^{125}$I-AGE-BSA binding) and AGE-ribonuclease contained 62 AGE U/mg.

To examine the effect of early glycosylation product reduction on ligand binding, AGE-BSA was incubated with 200 molar excess $NaBH_4$ (Sigma Chemical Co.) for 10 min at 4°C, followed by 1 h at room temperature. The reduced AGE-BSA was then dialyzed against 1× PBS, and the protein concentration was determined as above. The chemically defined AGE, 2-furoyl-4-(5)-(2-furanyl)-1-*H*-imidazole (FFI), was synthesized and linked to BSA with 100 mM water soluble carbodiimide as described previously (8).

*Iodination of AGE-BSA.* AGE-BSA was iodinated with carrier-free-$^{125}$I by the IODO-GEN method (Bio-Rad Laboratories) of Fraker and Speck (25). Samples were dialyzed against PBS until >95% of radioactivity was TCA precipitable and the samples were iodide free.

*Preparation of AGE Matrices.* Six-well plates coated with rat tail collagen, type 1, human fibronectin, and polylysine were purchased from Collaborative Research, Inc. (Bedford, MA). AGE matrices were produced by incubating the various matrix coated plates in 0.5 M G-6-P, at 37°C for 2–3 wk in 10 mM PBS buffer (pH 7.4), as described for AGE-BSA. Control matrices were incubated under identical conditions in buffer alone. After incubation, the plates were washed extensively with 1× PBS. The amount of adhered collagen I was determined using a hydroxyproline assay (26), while adhered fibronectin and laminin were determined by the method of Lowry et al. (27) after dissolving the matrix in 2 N NaOH at

37°C overnight, as described by Jones et al. (28), and by absorbance at 280 nm. In both cases, similar amounts of unmodified or AGE-modified matrix proteins adhered (collagen I, ~85%; fibronectin, ~70%; laminin, ~80% of the plated amount remained attached to the plates). AGE levels in matrix proteins were quantitated by an AGE-specific radioreceptor assay, as described above (11, 24) AGE-collagen I contained 47 AGE U/mg, AGE-fibronectin contained 54 AGE U/mg, and AGE-laminin contained 51 U/mg. Unmodified matrices contained <5 AGE U/mg protein.

*Membrane Preparation.* Rat and human MC were grown to confluency in 150-mm petri dishes. Cells were detached from the plates by PBS containing 3% EDTA and protease inhibitors (2 mM PMSF, 10 μg/ml aprotinin, 5 ng/ml pepstatin, and 1 mM 1,10-phenanthroline). After centrifugation, cells were disrupted with a tight Dounce homogenizer, pestle A, in a solution of PBS, with 10 mM EDTA and protease inhibitors, as stated above. The nuclear and organelle-enriched fractions were removed by centrifugation at 13,000 *g*. Membranes were then isolated from the supernatant by centrifugation at 100,000 *g* for 1 h at 4°C. The resulting enriched membrane fraction was solubilized in PBS, containing 1% Triton X-100 and 2 mM PMSF. The protein concentration was determined by the method of Bradford (22). This material was then used for binding and ligand blotting studies.

*Binding Studies.* Filter binding studies were performed according to the method of Schneider et al. (29) and Daniel et al. (30) with minor modifications. 10–20 μg of MC membrane protein was dot-blotted onto nitrocellulose filters. The nitrocellulose filters were then cut and each dot was placed in a separate well of a 24-well plate. After blocking of the filters for 1 h at 4°C in PBS containing 1.5% BSA, binding studies were initiated by adding various concentrations of radioactive ligand to the individual wells. At 2 h, the nitrocellulose filters were washed three times with ice-cold 1× PBS, and radioactivity bound was quantitated using a scintillation counter (Packard Tricarb, Paramus, NJ). Specific binding was defined as the difference between total binding (radioligand incubated with membrane protein alone), and nonspecific binding (cells incubated with radiolabeled ligand plus 100-fold excess unlabeled ligand). Scatchard analysis of the data was performed to determine the binding affinity constant and the receptor number, as described previously (31). Competition studies were performed in a similar manner to the binding studies, with the exception that the nitrocellulose filters were preincubated with the competitor for 1 h before adding the radiolabeled ligand.

Binding studies were also performed on confluent MCs in six-well plates. The studies were performed in 1 ml of RPMI 1640 at 4°C after the addition of various concentrations of radioactive ligand, as described previously (8) and above. After 2 h of binding, the radioligand-containing medium was removed, and the cells were washed with ice-cold PBS. The cell monolayer was then disrupted with 1% Triton X-100, and the cell-associated radioactivity was quantitated. Protein concentration was determined by the method of Bradford (22). Specific binding was determined in an identical manner to that described above for the filter binding assay.

*Ligand Blotting.* MC membrane preparations (5-μg aliquots) were subjected to electrophoresis on a nonreducing SDS-PAGE (10%), and then electroblotted onto a nitrocellulose filter, as previously described (32). After blocking for 1 h in a solution of PBS containing 1.5% BSA, the nitrocellulose filter was probed with $^{125}$I-AGE-BSA in the presence of 100-fold excess of either BSA or AGE-BSA. The blot was washed three times with 1× PBS and exposed to Kodak XAR-5 film at −80°C.

*Uptake and Degradation.* MC uptake and degradation was performed with a minor modification of previously described procedures (8). Briefly, MCs were grown to confluency in six-well plates in DMEM containing 20% FCS and insulin. MC accumulation of radioactive ligand (AGE-BSA) was assessed by incubating cells with various concentrations of $^{125}$I-AGE-BSA, in the presence and absence of 100-fold excess of unlabeled AGE-BSA, for 4 h at 37°C. After washing the cells three times with ice-cold PBS, the cells were solubilized in 1% Triton X-100 for 45 min at room temperature, and the amount of cell-associated radioactivity was determined. Specific uptake was defined by the same criteria as used for the MC binding studies. Protein concentration was determined by the method of Bradford (22). Degradation was determined by measuring TCA-soluble radioactivity in the aspirated medium.

*Proliferation Assays.* Rat MCs in DMEM containing 20% FCS were plated at $10^4$ cells/well onto flat-bottomed 96-well microtiter plates, which had been pre-coated with different amounts of either AGE-modified or unmodified matrix proteins. 24 h later, the cells were washed with 1× PBS and incubated for an additional 48 h in medium containing 0.3% FCS. The cells were then labeled with 2 μCi of [$^3$H]thymidine (Amersham Corp., Arlington Heights, IL) for 18–24 h, after which the supernatants were aspirated and the cells in each well were harvested onto glass fiber filters with an automated cell harvester. The amount of [$^3$H]thymidine incorporated was determined with a scintillation counter (Beckman Instruments). To confirm thymidine incorporation data, parallel studies were carried out using an immunocytochemical assay system for detection of DNA synthesis by measuring bromo-deoxyuridine (BrdU) incorporation (33), while in separate experiments, cells were trypsinized and counted in a particle counter (Coulter Electronics, Hialeah, FL). The data obtained by these two additional methods were consistent with [$^3$H]thymidine results (variations between replicate wells deviated no more than 10%).

*Fibronectin Production.* Human MCs in RPMI 1640 supplemented with 20% FCS were plated at $2 \times 10^5$ cells per well onto six-well plates that had been coated with glucose-modified or unmodified matrix proteins. After 24 h, the cells were washed with 1× PBS and incubated in medium containing 0.3% FCS for 48 h. The cells were then labeled in methionine-free medium for 3 h with 200 μCi of $^{35}$S-methionine and cysteine (Translabel; ICN, Plainview, NY). After labeling, the medium was removed and the cell monolayers were washed with cold 1× PBS. The monolayers were extracted with 0.5 ml of a 1-M urea solution containing 1 mM dithiothreitol (DTT), 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 2 mM PMSF, as previously described (34, 35). Fibronectin was then isolated from the medium and matrix by immunoprecipitation with an IgG purified anti–human fibronectin antibody (Cappel Laboratories, Malvern, PA). Antifibronectin antibody was added to the samples and incubated overnight at 4°C. To insure that any differences in fibronectin synthesis were not due to different number of cells/well, equal amounts of TCA-precipitable counts were immunoprecipitated from each well. The immune complexes were isolated using protein A–Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ). After washing the protein A–Sepharose beads three times in 100 mM Tris HCl (pH 7.4), 0.5% SDS, 0.5% Triton X-100, 2 mM PMSF, and 10 mM EDTA, fibronectin was released by heating at 100°C for 5 min in SDS-PAGE sample buffer, and analyzed by gel electrophoresis and fluorography. The amount of fibronectin from each sample was quantitated by slicing the fibronectin band from the gel and determining $^{35}$S-methionine and cysteine incorporation in a liquid scintillation counter.

## Results

*AGE-binding Site.* Membrane extracts isolated from both rat and human cultured MCs were found to contain binding

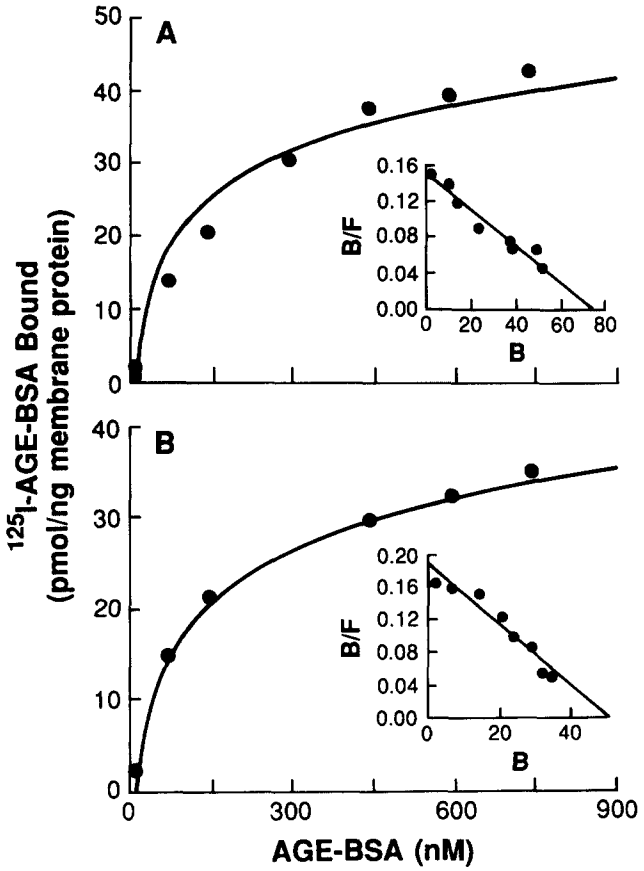


**Figure 1.** Binding of $^{125}$I-AGE-BSA to human (*A*) and rat (*B*) MC membranes. 10 $\mu$g of solubilized membrane protein from human and rat mesangial cells were dot-blotted onto nitrocellulose filters, which were then incubated with various concentrations of $^{125}$I-AGE-BSA in the presence and absence of 100-fold excess unlabeled AGE-BSA. Specific binding was obtained by subtracting the nonspecific binding from the total binding. The inset shows a Scatchard plot for the specific binding ($B$ = pmol/ng membrane protein, $F$ = nM).

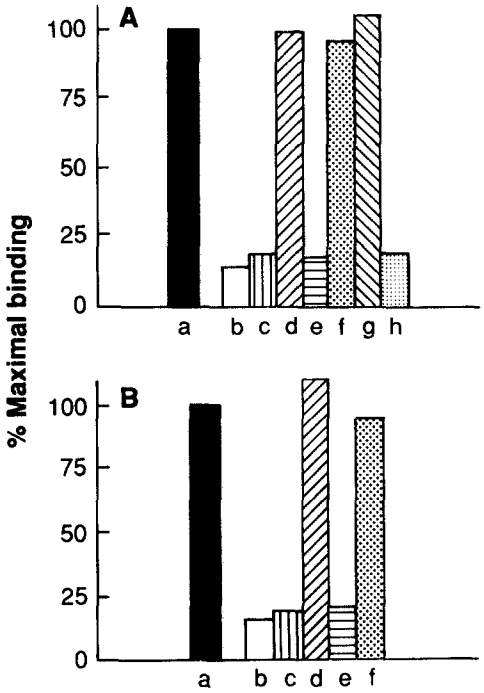


**Figure 2.** Competitive inhibition of $^{125}$I-AGE-BSA binding to human (*A*) and rat (*B*) MC membranes. 10 $\mu$g of solubilized membrane protein from human and rat MCs was dot-blotted onto nitrocellulose filters. The filters were incubated with 50 nM $^{125}$I-AGE-BSA for 2 h at 4°C. Competition experiments were performed in parallel experiments in which the radioligand was incubated with 100-molar excess of an unlabeled protein. Data shown are the average of duplicate determinations, and are expressed as the percent maximal binding. Maximal binding was defined as the amount of $^{125}$I-AGE-BSA bound in the presence of 100-molar excess cold BSA. Competitors used: (*a*) BSA, (*b*) AGE-BSA, (*c*) $NaBH_4$-reduced AGE-BSA, (*d*) FFI-BSA, (*e*) AGE-RNAse, (*f*) RNAse, (*g*) Collagen I, (*h*) AGE collagen.

sites for AGE-modified proteins, using AGE-BSA as a model radioligand (Fig. 1). When MC membrane extracts were incubated with increasing concentrations of $^{125}$I-AGE-BSA, specific binding increased in a saturable fashion. Half-maximal binding was observed at a concentration of AGE-BSA of ~150 nM. Analysis of the specific binding data by Scatchard analysis indicated that both rat and human MCs displayed similar binding characteristics. Assuming that each labeled AGE-BSA molecule can bind to only a single receptor site, the number of AGE molecules bound per cell ranged at 3.0 $\pm$ 0.25 $\times$ $10^5$ molecules per cell with a binding affinity constant of 2.0 $\pm$ 0.40 $\times$ $10^6$ $M^{-1}$ ($K_d$ = 500 nM). Similar results were also obtained when binding studies were performed on intact cells at 4°C (data not shown).

To confirm that AGE modification of BSA was responsible for binding onto MC surface, competitive inhibition experiments were performed using either rat or human MC membrane extracts (Fig. 2). These experiments confirmed that AGE modification of the protein was responsible for binding, since excess cold AGE-BSA (Fig. 2, *Ab* and *Bb*), but not unmodified BSA (Fig. 2, *Aa* and *Ba*), could competitively inhibit >80% of $^{125}$I-AGE-BSA binding to MC membrane extracts. In addition, other AGE proteins, including AGE ribonuclease (Fig. 2, *Ae* and *Be*) and AGE collagen I (Fig. 2 *Ah*), successfully inhibited $^{125}$I-AGE-BSA binding to MC membrane extracts. In sharp contrast, excess unmodified ribonuclease and collagen I failed to compete for binding (Fig. 2, *Af*, *Bf*, and *Ag*).

To eliminate the possibility that early glycosylation products were responsible for ligand binding, AGE-BSA preparations were subjected to reduction using $NaBH_4$. This agent effectively reduces the Shiff base and Amadori products to glucitollysine. The ability of reduced AGE-BSA to still compete effectively for $^{125}$I-AGE-BSA binding to MC membrane extracts suggests that the ligand binding activity is due to moieties other than Amadori products, such as intermediate and advanced glycosylation adducts (Fig. 2, *Ac* and *Bc*). The ability of the chemically synthesized AGE (FFI) to inhibit ligand binding was also assessed. While FFI-BSA has been shown to bind to the macrophage AGE receptor, no competitive inhibition was exhibited by FFI-BSA for $^{125}$I-AGE-BSA binding to MC membrane extracts (Fig. 2, *Ad* and *Bd*). In this context, the MC receptor appears different from the mac-

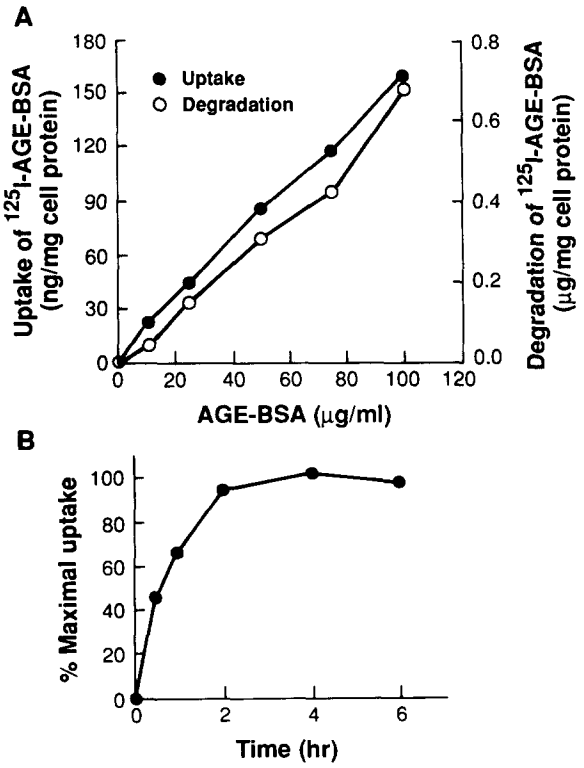


**Figure 3.** (*A*) Uptake and degradation of $^{125}$I-AGE-BSA by rat MCs. MCs were incubated with various concentrations of $^{125}$I-AGE-BSA for 4 h at 37°C. The amount of cell-associated $^{125}$I-AGE-BSA (uptake), and the amount of TCA-soluble counts in the medium (degradation) were determined in triplicate wells. (*B*) Accumulation of $^{125}$I-AGE-BSA vs. time. MCs in each well were incubated with 20 μg of $^{125}$I-AGE-BSA at 37°C, and specific cell-associated radioactivity was determined at various time intervals. Cellular accumulation of radioactivity is expressed as the percent of the maximal accumulation of $^{125}$I-AGE-BSA.

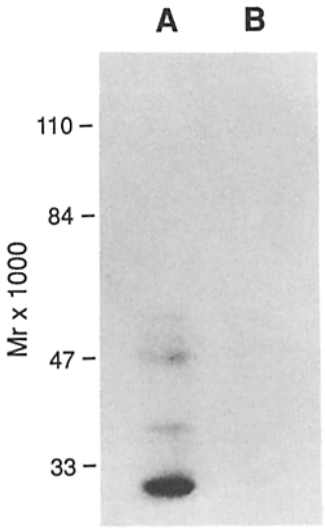


**Figure 4.** Ligand blot analysis of enriched human MC membranes. 10 μg of solubilized membrane protein was subjected to electrophoresis on a nonreducing SDS/polyacrylamide gel (10%). The proteins on the gel were electroblotted onto nitrocellulose membrane and probed with $^{125}$I-AGE-BSA in the presence of 100-fold excess of either BSA (lane *a*) or AGE-BSA (lane *b*). The analysis presented is one of four identical experiments.

rophage FFI-binding AGE receptor, and similar to the endothelial AGE receptor, which does not recognize FFI-BSA (13).

*AGE Uptake and Degradation by MC at 37°C.* To determine whether MCs can internalize and degrade AGE proteins, cells were incubated with $^{125}$I-AGE-BSA at 37°C, and cell-associated radioactivity was measured with respect to AGE-BSA concentration and length of incubation (Fig. 3, *A* and *B*). When MCs were incubated at 37°C with increasing concentrations of $^{125}$I-AGE-BSA, specific cell-associated radioactivity increased with increasing concentrations of radioactive ligand (Fig. 3 *A*). However, in contrast to the binding data, which exhibited saturable kinetics, MCs at 37°C continued to specifically accumulate AGE-BSA even at concentrations as high as 1.1 μM. Since the accumulation of AGE-BSA at 37°C could represent both bound as well as internalized ligand, we compared accumulation of AGE-BSA at both 37°C and 4°C. Maximal accumulation of $^{125}$I-AGE-BSA occurred within 2 h of incubation at 37°C (Fig. 3 *B*), while the amount of cell-associated radioactivity at 37°C was two- to fourfold higher than that bound at 4°C. This, coupled with the different saturation kinetics, is consistent with the participation of MCs in both the binding and internalization of AGE-BSA.

Concomitant with the MC accumulation of $^{125}$I-AGE-BSA, ligand degradation also increased, as measured by a steady increase in TCA-soluble radioactivity in the media. The increase in AGE-BSA degradation paralleled the increase in MC uptake of AGE-BSA (Fig. 3 *A*).

*Ligand Blot.* To confirm and characterize better the AGE-binding sites on MCs, ligand blotting studies were performed using detergent extracts of MC membranes. As shown in Fig. 4, three prominent AGE-binding proteins of 50, 40, and 30 kD were consistently observed. Binding of radiolabeled AGE to these membrane proteins was specific, since excess unlabeled AGE-BSA, but not BSA alone, could completely inhibit binding. The results were identical for both human and rat mesangial cells (human MC data shown). While it is possible that the lower molecular mass bands represent degradation products, the isolation of membranes at 4°C in the presence of a variety of protease inhibitors, as well as the reproducibility of results between experiments, argues against significant degradation.

*Inhibitory Effect of AGE Matrices on [$^3$H]Thymidine Incorporation by MC.* The effects of unmodified and AGE-modified matrix proteins on MC proliferation were assessed (Fig. 5). MCs grown on plates containing 10 μg/ml unmodified fibronectin or collagen I exhibited greater [$^3$H]thymidine incorporation than cells plated on plastic. AGE modification of these matrices, when used at a concentration of 10 μg/ml, resulted in a consistent decrease in [$^3$H]thymidine uptake approximating the levels of incorporation of cells plated on plastic. While unmodified laminin substrates did not stimulate the uptake of [$^3$H]thymidine over control values, AGE modification of laminin was associated with a decrease in [$^3$H]thymidine incorporation. This decrease was less marked than that observed after AGE modification of fibronectin and collagen IV. The observed differences in [$^3$H]thymidine uptake were confirmed by BrdU incorporation assays, used to control for DNA synthesis (33), and were not attributable

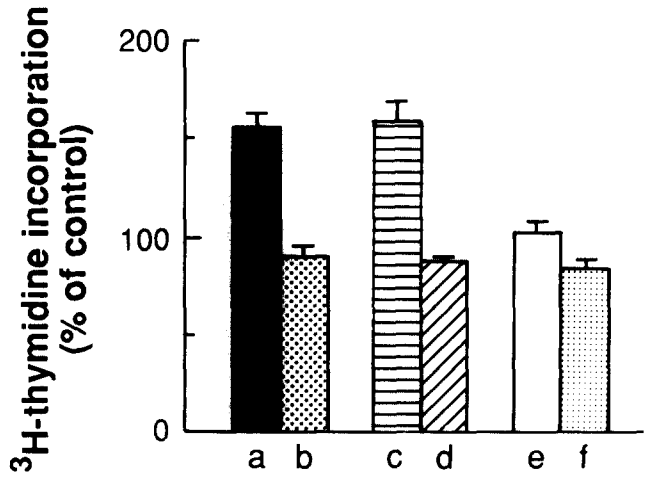


**Figure 5.** Effects of AGE-matrices on [$^3$H]thymidine incorporation by MCs. Rat MCs were plated onto various matrices (10 $\mu$g/ml), as described: (*a*) fibronectin, (*b*) AGE-fibronectin, (*c*) collagen I, (*d*) AGE-collagen-I, (*e*) laminin, (*f*) AGE-laminin. The results are expressed as the means ± SEM of six experiments and are expressed as the percent of [$^3$H]thymidine incorporated relative to the control value, with control representing [$^3$H]thymidine incorporated by cells plated on plastic.

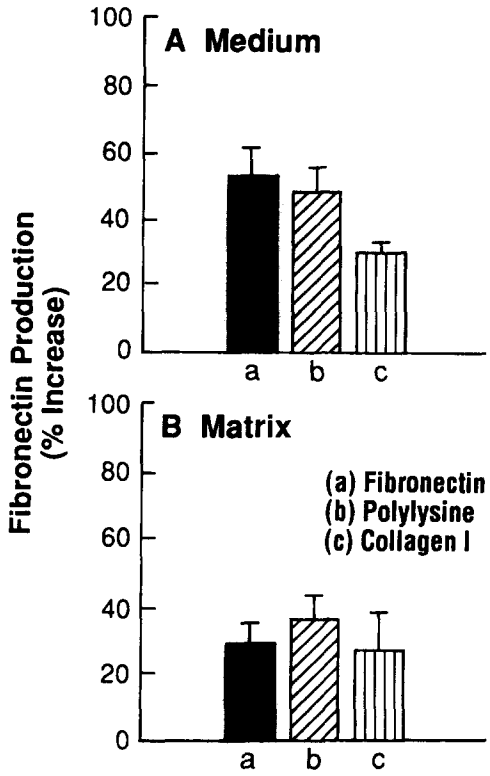


**Figure 6.** Effect of AGE matrices on fibronectin synthesis by MCs. Human MCs were plated onto either unmodified or AGE-modified matrices and labeled with $^{35}$S-methionine and cysteine, as described. The amount of fibronectin released into the medium (*A*), and incorporated into the matrices (*B*), was determined by immunoprecipitation. The fibronectin bands on the gel were excised and counted for radioactivity. The values shown are expressed as the percent increase in fibronectin produced by cells plated on the AGE matrices relative to that produced by cells plated on control unmodified matrices. The values show cpm/well and represent the means ± SEM from four experiments. (*a*) Fibronectin, (*b*) polylysine, (*c*) collagen I.

to varying numbers of MCs adhering to the individual matrices; cell counting of adherent MCs 8 h after plating revealed that a similar number of MC adhered to each matrix.

*AGE Matrices Stimulate Fibronectin Production.* To determine whether MC interaction with AGE-modified matrices could enhance the production of fibronectin, MCs were plated onto either AGE-modified or unmodified collagen I, fibronectin, and polylysine. Quantitative analysis of adhered modified or nonmodified matrix components, e.g., collagen I and fibronectin, indicated that identical amounts adhered onto the plastic surfaces. Fibronectin released into the medium or incorporated into the matrix was quantitated by immunoprecipitation followed by PAGE, fluorography, and radioactivity determination as described in Materials and Methods. MCs grown on the AGE-modified matrices exhibited an increase in fibronectin secretion (Fig. 6). MCs grown on AGE collagen I, AGE fibronectin, and AGE polylysine released 33 ± 3.6%, 54 ± 6.6% and 48 ± 7.7% more fibronectin, respectively, into the medium, compared to MCs grown on unmodified matrices (Fig. 6 *A*). MCs grown on AGE matrices also incorporated more fibronectin into the matrix than cells grown on unmodified matrices (Fig. 6 *B*).

## Discussion

The results of this study indicate that both human and rat MCs express binding sites that selectively recognize glucose-modified proteins. Evidence in favor of the existence of these sites includes several findings: MC membranes are able to bind AGE-BSA, a model ligand, in a saturable fashion, exhibiting half-maximal binding at a concentration of AGE-BSA of ~150 $\mu$M. Binding of AGE-BSA to MCs is specific for the AGE adduct, since several AGE-modified proteins, but not unmodified proteins, compete for binding. In addition, $NaBH_4$-reduced AGE-BSA effectively competes for binding of AGE-BSA to MCs. This argues against (although not completely excludes) the notion that the binding domain is related to an early product of nonenzymatic glycosylation, since the treatment of AGE-BSA with $NaBH_4$ reduces both the Schiff base and Amadori products to glucitollysine. Finally, ligand blotting studies confirmed the presence of three membrane proteins with molecular masses of ~50, ~40, and ~30 kD that specifically bind AGE-BSA.

In addition to binding AGE-BSA, MCs also appear to participate in both the uptake and degradation of AGE-BSA at 37°C. This is supported by several lines of evidence. First, MCs exposed to radiolabeled AGE-BSA at 37°C accumulate two- to fourfold more radioactivity than cells incubated at 4°C for the same period of time. Further evidence for the internalization of AGE-BSA is derived by comparison of the binding curves at 4°C and 37°C. At 4°C, MCs bind AGE-BSA in a saturable fashion. However, at 37°C, MCs continue to accumulate $^{125}$I-AGE-BSA in a nonsaturable manner up to concentrations of AGE-BSA as high as 1.1 $\mu$M. This increase in cell-associated radioactivity at 37°C could be attributed to slow internalization of the AGE receptor complex with subsequent recycling of the receptors back to the cell surface after the intracellular release of AGE-BSA. A similar

mechanism has recently been reported for the MC LDL receptor (36). Finally, MCs were found to degrade AGE-BSA slowly at 37°C, with kinetics that paralleled those of AGE-BSA uptake. While the possibility remains that degradation of AGE-BSA occurs as a result of secreted proteases, the close association between MC accumulation and degradation of AGE-BSA supports the participation of MC receptors in internalizing and degrading AGE-BSA.

This ability of MCs to slowly internalize and degrade AGE proteins points to a contributing role for MCs to the in vivo turnover and remodeling of senescent AGE-modified mesangial matrix proteins. However, the efficiency with which MCs ingest and degrade AGE-BSA is only comparable to 10% of that which has been reported for the macrophage (8). In view of the fact that the kidney mesangium contains blood-derived macrophages as well as kidney MCs (37), the relative contribution of each cell type to the in vivo turnover of senescent mesangial proteins may differ considerably, and remains to be defined.

The relationship between this AGE receptor identified on the MC and the AGE receptor reported on monocytes/macrophages and ECs has not been completely clarified. Within a given cell lineage, the AGE receptor is highly conserved between mouse, rat, and human cells. Functionally, both the rat and the human MC AGE receptors exhibit similar binding characteristics, and structurally both yield AGE-binding proteins of similar molecular mass as estimated by ligand blot studies. Likewise, the murine and human macrophage AGE receptors display similar structural and functional characteristics (38, 39). However, while the AGE receptors on all three cell types are similar in that they all recognize advanced glycosylation products, several differences exist (8, 13). The $K_d$s for the receptors are different; the $K_d$s for the macrophage and EC AGE receptor are ~70–100 nM, while the $K_d$ for the MC is about fivefold higher. In addition, the efficiencies with which these receptors internalize and degrade AGE proteins are different. Macrophages ingest and degrade AGE-BSA at a much higher rate than either ECs or MCs. Finally, the macrophage AGE receptor recognizes the specific adduct FFI, which is not recognized by either the MC receptor or the EC receptor. These differences between AGE receptors of different cell types suggest that distinct subclasses of the receptor exist. In the case of MCs, preliminary structural evidence suggests that these receptors consist of a diverse group of peptides of different molecular masses and possibly function.

To determine whether the interaction of MCs with AGE-modified matrix proteins could influence MC function, MCs were plated onto AGE matrices. Since MC hyperplasia has been reported in the diabetic kidney, we were first interested in assessing whether plating MCs on AGE matrices could stimulate MCs to proliferate. In contrast to the increase in MC number which has been reported in the diabetic kidney (3), MCs plated on AGE matrices exhibited a decrease in proliferation as measured by [$^3$H]thymidine incorporation. Crowley et al. (40) also found that MC proliferation was inhibited when MCs were grown on MC-generated matrices that had been nonenzymatically glycosylated with glycoaldehyde. One possibility that could account for the discrepancy between this in vitro result and in vivo findings is that MC proliferation in vivo is not mediated by a direct interaction of AGE matrix proteins with MCs. In vivo, the interaction of MCs with other components of the glomerular mesangium, such as blood-derived macrophages, could provide a paracrine proliferative stimulus to MCs in the diabetic kidney. The interaction of macrophages with AGEs has been shown to induce the release of a variety of cytokines, such as cachectin/TNF and IL-1, which could have paracrine growth-promoting effects on MC in vivo (14).

Increased mesangial accumulation of fibronectin has been noted in humans and experimental animals with diabetic kidney disease (4, 5). In addition, the presence of elevated fibronectin mRNA levels in kidneys from diabetic animals (41) indicates that enhanced synthesis of fibronectin accounts for at least part of the increased accumulation. The observation that the interaction of MCs with AGE matrices in vitro induces an increase in fibronectin secretion suggests that in vivo, the interaction of MCs with AGE-modified matrices may stimulate MCs to enhance synthesis of this important matrix component.

Recent experimental findings have documented the importance of ambient glucose concentration in modulating cell function (42–44). Incubation of MCs in high glucose medium leads to a diminished rate of proliferation, along with increased production of several matrix proteins, including fibronectin, collagen IV, and laminin. The findings reported here provide an additional mechanism by which elevated glucose levels, acting through AGE modification of matrix proteins, may alter MC function. In addition, it is possible that some of the functional alterations detected in the elevated glucose medium may actually have resulted from AGE modification of existing matrix proteins, since a time delay of several days in high glucose medium was necessary before alterations in MC function became apparent.

The upregulation of fibronectin mRNA in kidneys from diabetic animals has been found to persist for several weeks after the restoration of normoglycemia (41). Since the formation of AGEs occurs in an irreversible fashion, it follows that the "memory" of a prior elevated glucose concentration should persist in the form of AGE moieties within long-lived matrix proteins. The continued interaction of these altered proteins with MCs, despite restoration of normoglycemia, may be one of the stimuli for MCs to continue producing fibronectin. This finding may also help to explain the difficulty encountered in treating clinical diabetic kidney disease. Once kidney disease has occurred, controlling of ambient glucose concentration does little to avert the progression of disease (45). While many mechanisms undoubtedly contribute to this phenomenon, the continued interaction between AGE-modified matrices and kidney MCs may contribute to the progressive functional abnormalities, despite normalization of blood glucose levels.

At present it is not clear whether the alteration in MC function associated with exposure to AGE matrices is mediated via the MC AGE-binding sites. In this regard, we have been unable to demonstrate these functional alterations using a soluble AGE ligand. However, it is known that the ability

of a ligand to crosslink its receptor may be critical for signal transduction (46). While soluble ligands may not be able to perform this function, ligands adhering to a cell surface acquire the ability to crosslink their receptor. This phenomenon could provide one mechanism whereby AGE matrices could interact with AGE receptors, while soluble AGE ligands can not.

Alternatively, it is possible that the formation of AGEs on matrix proteins may affect MC function by altering normal matrix-matrix and matrix-cell interactions distinct from the AGE receptor pathway. The formation of AGEs on matrix proteins results in protein crosslinking that could cause the distortion of important protein recognition sites through mechanisms unrelated to the AGE receptors. The binding of anionic proteoglycans, such as heparin sulfate, to matrix proteins is reduced after AGE modification (47). Similarly, the ability of fibronectin to bind collagen is impaired after AGE modification (48). These effects of glycosylation may alter the composition of matrix. Matrix proteins are also able to interact with distinct receptors on MCs, and this interaction is important in the regulation of cell adhesion, cell growth, and gene expression (49). Glycosylation of matrix proteins may alter their recognition by cellular receptors. It is therefore conceivable that nonenzymatic glycosylation of normal matrix proteins, by altering normal matrix composition and/or distorting normal cellular recognition sites for matrix proteins, may produce some of the functional changes observed in MC phenotype.

The studies reported here represent the identification and initial characterization of mesangial cell surface binding proteins that recognize AGE proteins, and provide a mechanistic basis whereby AGE-modification of matrix proteins in the kidney may lead to alterations of MC function. We demonstrated that MCs plated on AGE-modified matrices exhibit altered functional characteristics, including enhanced production of fibronectin and decreased proliferation. Many questions remain to be addressed; in particular, the relationship between AGE receptors reported on different cell types, and the relationship of the MC AGE-binding proteins to other known MC receptors remain to be elucidated. We can now begin to explore the range of biological effects that may be modulated via these molecules, and to investigate the in vivo role that they may play in maintaining normal kidney homeostasis. Finally, we can begin to assess the degree to which these receptors may contribute to the development of diabetic renal complications.

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