Receptor-specific increase in extracellular matrix production in mouse mesangial cells by advanced glycosylation end products is mediated via platelet-derived growth factor

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Communicated by Anthony Cerami, December 26, 1991 (received for review December 2, 1991)

ABSTRACT Renal disease is one of the most common and severe complications of diabetes mellitus. The hallmark of the disease, glomerulosclerosis, is characterized by an accumulation of extracellular matrix in the mesangial areas, leading to progressive obliteration of the vascular spaces. The role of the metabolic derangements of diabetes mellitus in the development of these lesions is incompletely understood. One of the consequences of hyperglycemia is the formation of advanced glycosylation end products (AGEs), which result from a series of rearrangements secondary to nonenzymatic reaction of glucose with proteins. Specific receptors for proteins modified by AGEs, present in several cell types, were recently described in human and rat mesangial cells. Furthermore, exposure of mesangial cells to AGEs was followed by an increase in fibronectin production. In the present study we show evidence that mouse mesangial cells exhibit an increase in collagen type IV mRNA and peptide synthesis after exposure to AGEs. Antibodies to AGE receptors prevent this increase, indicating that the response is AGE-receptor-mediated. In addition, anti-platelet-derived growth factor abrogates the AGE response, suggesting that platelet-derived growth factor acts as an intermediate factor. Transcription assay reveals that the elevated mRNAs levels are due to an increase in the transcription rate, rather than to an increase in the stability of the message. Finally, the mRNAs coding for laminin and heparan sulfate proteoglycan are also increased after exposure to AGE, whereas glyceraldehyde 3-phosphate dehydrogenase mRNA levels remain constant. The increase in extracellular matrix mRNAs seen in the current study suggests that AGE formation in vivo may be one of the metabolic events leading to the development of diabetic glomerulosclerosis.

The development of advanced glycosylation end products (AGEs), resulting from nonenzymatic glycosylation of proteins, is believed to play a role in the development of diabetic nephropathy (1, 2). The hallmark of the disease, known as glomerulosclerosis, is an accumulation of extracellular matrix (ECM) components that gradually obliterate the glomerular vascular loops and cause kidney failure in 30–40% of diabetics (3). Morphological studies suggest that the lesions originate within the mesangial regions, leading to the postulate that AGEs may affect mesangial cell functions (4, 5). This concept is strengthened by the recent finding that mesangial cells from human and rat glomeruli have AGE-specific receptors (4). We have developed a line of normal mouse mesangial cells, which, unlike human and rat mesangial cells in vitro, synthesize predominantly the basement membrane components found in intact glomeruli in vivo (6). In the present study using this cell line, we found that AGEs lead to an up-regulation of transcription, translation, and secretion of type IV (basement membrane) collagen. This effect appears to be receptor-mediated because it is inhibited by antibodies raised against receptors for AGEs. The AGE-induced increase in type IV collagen message is also abrogated by cell exposure to platelet-derived growth factor (PDGF) antibody. Thus, AGEs may play a role in the increase in ECM that characterizes the glomerular lesion of diabetic nephropathy. The role of advanced glycosylation end products (AGEs) may be accentuated by atherosclerosis (7), which is a chronic inflammatory disease (8). AGEs may also be a contributing factor in the development of diabetic nephropathy (9).

MATERIALS AND METHODS

Cell Culture. A glomerular mesangial cell line established from glomeruli isolated from normal 4-week-old mice (C57BL/6J × SJL/J) was used (7). Mesangial cells were identified as described (7). Phenotypically stable cells, passage 14–24, were plated in 100-mm plastic dishes (Nunc), maintained in B medium (minimal essential medium/F12 modified with trace elements, 3:1) supplemented with 6 mM glucose, 1 mM glutamine, penicillin at 100 units/ml, streptomycin at 100 μg/ml, and 20% fetal bovine serum (GIBCO) and passaged weekly with trypsin-EDTA.

Preparation of Ligands. AGE–bovine serum albumin (BSA) was prepared by incubating BSA (fraction V, low-endotoxin; Boehringer Mannheim) in phosphate-buffered saline (PBS) (10 mM, pH 7.4) with 50 mM glucose 6-phosphate or 0.5 M glucose at 37°C for 6 weeks with protease inhibitors and antibiotics as described (2, 4). Unincorporated sugar was removed by dialysis against PBS. Unmodified BSA and bovine ribonuclease (Sigma) were incubated under the same conditions without glucose or glucose 6-phosphate as controls. AGE–BSA and unmodified BSA were purified through heparin-Sepharose CL-6B (Pharmacia LKB) to remove contaminants. Endotoxin content in all samples was measured by Limulus amoebocyte lysate assay (E-toxate, Sigma) and found to be <0.2 ng/ml. AGE content was assessed by a radioreceptor assay for AGEs (8). AGE–BSA contained 55 AGE units per mg of protein, and unmodified BSA contained 0.42 AGE units per mg of protein.

Binding Assay. AGE–BSA was iodinated with carrier-free-125I by the Iodo-Gen method (Bio-Rad) as described (9). Mouse mesangial membranes were isolated from confluent

Abbreviations: AGE, advanced glycosylation end product; PDGF, platelet-derived growth factor; BSA, bovine serum albumin; ECM, extracellular matrix; GADPH, glyceraldehyde 3-phosphate dehydrogenase; HSPG, heparan sulfate proteoglycan. To whom reprint requests should be addressed at: Building 10, Room 3N110, Renal Cell Biology Section, Metabolic Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892.
mesangial cells as described in detail (4) and solubilized in PBS/1% Triton X-100/2 mM phenylmethylsulfonyl fluoride. Protein concentration was determined by the method of Bradford (10). Ten to twenty micrograms of membrane protein was dot-blotted onto nitrocellulose filters (4). After blockade with 1.5% BSA/PBS at 4°C for 1 hr, filters were incubated with various concentrations of radioactive ligand at 4°C for 2 hr with or without 100-fold excess of unlabeled ligand (AGE-BSA) as described (4), washed, and counted for 121I. Specific binding was defined as the difference between total binding and binding in wells containing excess unlabeled ligand (nonspecific binding). Scatchard analysis of data was used to determine the binding affinity constant (11).

Identification of Mesangial Cell AGE Receptors by Immunofluorescence. The presence and distribution of AGE receptors on mesangial cell surface was assessed by immunofluorescence microscopy by using polyclonal avian antibodies raised against two rat liver AGE-binding proteins, p60 and p90, as described (12). Ten thousand mesangial cells cultured on fibronectin-coated eight-well chamber slides (Nunc) for 24 hr were fixed at 4°C for 15 min with 2% paraformaldehyde and incubated with the following solutions: cold PBS containing 10% normal goat serum for 10 min, chicken anti-p60 or anti-p90 at 200 μg/ml (12) for 1 hr, biotin-labeled goat anti-chicken IgG (Vector Laboratories) and then by fluorescein isothiocyanate-streptavidin (Zymed Laboratories). The specificity of these antibodies was verified by Western (immuno) analysis, although these antibodies have been used to demonstrate expression of such receptors on rat blood monocytes, peritoneal macrophages, as well as murine RAW 264.7 cells (12). Nonspecific chicken IgG (Sigma) followed by biotin-labeled goat anti-chicken IgG was used as negative control.

RNA Isolation and Northern (RNA) Blot Analysis. Mesangial cells (1.2 × 106) were plated in medium/20% fetal bovine serum onto 100-mm dishes coated with AGE–BSA or control BSA at 5 μg/cm2. Preliminary experiments revealed that the AGE-induced effect was not detectable up to 48 hr of incubation with AGEs; therefore, subsequent experiments were done after 72 hr of exposure. The cells were washed with PBS, and total RNA was extracted by using the guanidine isothiocyanate method (13). Five micrograms of RNA was separated on a 1% formaldehyde/agarose gel and transferred to Nytran (Schleicher & Schuell). Filters were processed and analyzed as described (6).

RNA Probe Preparation. The cDNA (p1234) of Ava I from pFAC in pGEM2 (Promega) codes for a portion of the major triple helical and the globular domain (NC1) of the mouse α1(IV) collagen chain (14), pGM101, p1296, and pGMPG1 code for α2(I) procollagen, laminin B2, and mouse heparan sulfate proteoglycan (HSPG) (clone 5), respectively (6). The cDNA clones of Sts I–EcoRI site [1.15 kilobase pairs (kbp)] from mouse laminin A (p1238) and HindIII–EcoRI site (4.6 kbp) from mouse laminin B1 (p1235) were subcloned into pGEM2 (Promega) (15, 16). Orientation of plasmid inserts was analyzed by determining the size of digestible fragments with restriction enzymes.

Radiolabeled hybridizing probes were prepared by linearizing the constructs with Ava I (pGM101), Pvu II (p1234), Ava I (p1238), Nco I (p1235), HindIII (1298), and Xho I (pGMPG1). Before transcription, the linearized pGM101 and p1238 were treated with Klenow DNA polymerase (Promega) to convert the 3′ protruding ends to blunt termini. The transcription reaction was done according to Melton by using 1 mg of template DNA, [32P]UTP (Amersham), and the T7 or T4 RNA polymerase (Promega) as described (6, 17). The 1-kilobase (kb) ladder or 123-base pair (bp) ladder, which was labeled by the replacement synthesis method with T4 DNA polymerase (BRL/Life Technologies), was used as a control size marker. All restriction enzymes were purchased from BRL.

Solution Hybridization RNase Protection Assay. The RNA probe (5 × 105 cpm) and total RNA were hybridized overnight at 45°C. RNase A (40 μg/ml) and RNase T1 (2 μg/ml) were added to each tube and incubated for 1 hr at 30°C. RNase-resistant fragments were analyzed by 3.5% polyacrylamide/8 M urea gel electrophoresis and autoradiography (6). The protected band of each RNA probe had the same size as the coding sequence for specific mRNA, providing evidence for their specificity.

Transcript Stability. The stability of type IV collagen and GAPDH mRNAs in control and AGE-treated cells was assessed by incubating cells in 60 μM 5,6-dichloro-1-β-D-ribofuransosylbenzimidazole to block transcription initiation (18). After incubation for various times, total RNA was isolated and assayed as above.

Run-Off Transcription Assay. Nuclei were isolated from 5 × 106 cells as described (19), resuspended in buffer (50 mM Tris, pH 7.9/5 mM MgCl2/0.1 mM EDTA/30% glycerol) at 2 × 106 nuclei per μl, and aliquots were incubated for 30 min at 30°C with 10 mM Tris buffer, pH 7.9/250 μCi of [32P]UTP (1 Ci = 37 GBq, Amersham)/1 mM ATP/1 mM GTP/1 mM CTP (Pharmacia LKB)/5 mM MgCl2/300 mM KC1/RNasin at 2 units per μl. The reaction products were treated with RNase-free DNase for 16 min at 16°C and incubated with proteinase K at 200 μg/ml/0.1% SDS for 20 min at 37°C. Samples were extracted twice with phenol/chloroform and once with chloroform; free transcripts were removed with a Centricon 30 microconcentrator (Amicon). Denatured DNA [4 μg of a 0.85-kbp Pst I–Ava I fragment of α1(IV) collagen cDNA, a 1.3-kbp Pst I fragment of GAPDH probe, and 1 μg of pGEM2 plasmid as a negative control] were immobilized on Nitran. Filters were prehybridized and hybridized for 3 days at 65°C with equal counts of the radiolabeled RNA in 50 mM Hepes buffer, pH 7.4/0.3 M NaCl/10 mM EDTA/10 mM Tris at 1 mg/ml/poly A at 1 mg/ml/0.2% SDS. Filters were washed with 2× standard saline citrate at 65°C and incubated with RNase A at 10 μg/ml for 30 min at 37°C. The filters were then rinsed, and autoradiographs were prepared.

Effect of Anti-AGE Receptors and of Anti-PDGF Antibodies on Type IV Collagen mRNA Expression. Mesangial cells (5 × 106) were plated in six-well plates, coated with AGE–BSA or control BSA, in medium/20% fetal bovine serum. Antibodies to p60 and p90 (12) at a total concentration of 120 μg/ml were added to each well at plating in two separate experiments. After 72 hr, total RNA was extracted, and mRNAs were analyzed as above.

A polyclonal goat antibody raised against PDGF, which recognizes all three dimeric forms of PDGF (AB, AA, and BB) (Collaborative Research) was added to wells containing 5 × 105 cells in medium/20% fetal bovine serum at a final concentration of 50 μg/ml in two separate experiments. After 72 hr the RNA was extracted and analyzed as above.

Secretion of Type IV Collagen. Mesangial cells were plated in 100-mm dishes coated with AGE–BSA or control BSA in medium supplemented with 20% fetal bovine serum and maintained for 3 days. The wells were rinsed with serum-free medium and incubated with medium/0.1% BSA/1-α-ascorbic acid at 50 μg/ml. After 24 hr the medium was removed, and inhibitors were added to reach final concentrations of 25 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 mM N-ethylmaleimide.

Mouse type IV collagen was prepared from Engelbreth-Holm–Swarm tumor (20), and rabbit anti-mouse type IV collagen (HK68) was from Hynda K. Kleinman (National Institutes of Health). Type IV collagen was quantitated by enzyme-linked immunoadsorption using immunoplates coated with type IV collagen at 2 μg/ml.
RESULTS

AGE-Binding Sites on Mouse Mesangial Cells. Rat and human cultured mesangial cells have recently been shown to contain binding sites for AGE-modified proteins (4). To ascertain the presence of similar sites on mouse mesangial cells, membrane extracts from mouse glomeruli were tested for AGE-specific binding activity as described (4). With increased concentrations of 125I-labeled AGE-BSA, specific binding increased in a saturable fashion. Analysis of binding data indicated 2.25 ± 0.75 × 10^9 molecules per cell with a binding affinity constant of 1.75 ± 0.6 × 10^6 M⁻¹ (data not shown). As for rat and human mesangial cells (4), unmodified BSA or ribonuclease could not compete for binding with labeled AGE–BSA (data not shown).

To examine the ability of the mesangial AGE-binding sites to be recognized by antibodies raised against rat liver AGE-binding proteins p60 and p90 (12) and to visualize their distribution on the cell surface, mesangial cell monolayers were examined by immunofluorescence microscopy by using polyclonal avian anti-p60 and anti-p90 antibodies (12). These antibodies have been shown to block binding of AGE proteins, to screen for AGE-receptor expression on rat monocytes and peritoneal macrophages (12), and to inhibit AGE-induced synthesis of insulin-like growth factor I in human monocytes (21). With anti-p60 and anti-p90 antibodies as anti-AGE-receptor antibodies and chicken IgG as an irrelevant control, mesangial cell layers were examined by immunofluorescence microscopy. Many finely punctate dots were revealed over the entire surface of cells stained with either anti-p60 (Fig. 1A) or anti-p90 antibody (Fig. 1B), a distribution thought to represent coated pits on the plasma membrane. In contrast, cells stained with a nonspecific anti-IgG antibody remained completely negative (data not shown).

mRNA Levels of ECM Components. The mRNAs for collagen type IV, laminin A, laminin B1, laminin B2, and HSPG were readily detectable in all cells incubated with or without AGES, whereas collagen type I mRNA was not detectable at any of the passages examined in either cell group tested (Fig. 2). Three days after plating on AGE–BSA-coated wells mRNA levels increased for collagen type IV (1.52 ± 0.22 -fold, n = 9), compared with cells plated on BSA alone. This difference was not detectable at day 1 and disappeared at 7 days (data not shown). In addition, cells plated on AGE–BSA showed an increase in level of mRNA coding for laminin A (1.54 ± 0.16 -fold, n = 3), laminin B1 (2.42 ± 1.14 -fold, n = 3), laminin B2 (1.77 ± 0.39 -fold, n = 3), and HSPG (1.82 ± 0.32 -fold, n = 3). A GAPDH mRNA level was comparable in cells plated on control and AGE–BSA (1.04 ± 0.02 -fold, n = 3).

Addition of anti-AGE receptor antibodies (anti-p60 plus anti-p90) prevented the AGE-induced collagen type IV mRNA increase above control levels in two separate experiments, showing that the AGE-receptor system was involved in mediating the response (Fig. 3A). In contrast, addition of nonspecific chicken IgG had no effect on collagen IV mRNA levels in either AGE-stimulated or control cells (data not shown). The modest increase of collagen IV mRNA above control levels in the presence of BSA and anti-AGE-receptor antibody (Fig. 3A, lane 3) may suggest partial antibody agonist activity, although this effect was not reproducible.

Antibody to PDGF Inhibits AGE-Receptor Effect on Collagen Type IV mRNA Levels. Addition of antibody that recognizes the three dimeric forms of PDGF blunted the AGE-induced increase in collagen type IV mRNA seen at 72 hr but did not affect the level in cells plated on unmodified BSA (AGE: 1.98 ± 0.47 -fold over unmodified BSA, AGE plus anti-PDGF antibody: 0.67 ± 0.02, and unmodified BSA plus anti-PDGF antibody: 0.88 ± 0.04) (Fig. 3B).

Transcription Assay and Transcript Stability. A 2.18 ± 0.08 -fold increase in the transcription rate of collagen α1(IV) gene was seen in cells plated on AGE–BSA (Fig. 4A). The GAPDH transcription rate did not differ between control or experimental nuclei. Thus, one contributor to the increased collagen α1(IV) mRNA was an increase in its transcription rate. There were no differences between the stability of collagen α1(IV) and GAPDH mRNAs in control cells and those plated

Statistical Analysis. Data were analyzed by the unpaired Student’s t test and expressed as the mean ± SEM.

Fig. 1. Distribution of AGE receptors on mesangial cells by immunofluorescence with anti-AGE receptor antibodies. (A) Anti-p60. (B) Anti-p90.

Fig. 2. Effect of AGE on mRNA levels expressed by mesangial cells. mRNAs coding for ECM were measured by solution hybridization protection assay and mRNA coding for GAPDH by RNA blot analysis. Cells were exposed to control BSA (lanes 1, 3, 5, 7, 9, 11, 13) or to AGE–BSA (lanes 2, 4, 6, 8, 10, 12, 14). Data are representative of several experiments. Lam, laminin.
on AGE-BSA, ruling out a role for decreased degradation of collagen α1(IV) mRNA in the AGE-induced effect (Fig. 4B).

**Secretion of Type IV Collagen.** To determine whether the observed increases in collagen IV mRNA synthesis and transcription rates were associated with a parallel increase in protein secretion, collagen IV mRNA levels were quantitated in the medium of AGE-stimulated mesangial cells and compared with normal. A 1.34 ± 0.04-fold increase in amount of type IV collagen was detected in the medium of cells plated on AGE-BSA compared with cells plated on native albumin (Fig. 5).

**DISCUSSION**

Mouse mesangial cells plated onto culture dishes coated with AGE-modified BSA exhibited an increase in collagen type IV, laminin A, laminin B1, laminin B2, and heparan sulfate proteoglycan mRNA levels. The up-regulation of matrix component mRNAs was not a generalized cellular event, as evidenced by the stable levels of the GAPDH mRNA. The increased collagen type IV mRNA levels were associated with an increase in transcription rate, whereas the stability of this mRNA was not changed. Finally, the AGE-induced increase in mRNA levels resulted in increased secretion of collagen type IV into the medium, consistent with our previous observations that exposure of mesangial cells to AGEs leads to an increase in fibronectin production (4).

Thus, exposure of mesangial cells to AGE proteins induced increased synthesis and export of ECM components.

We have established that mouse mesangial cells have AGE receptors similar to those on rat and human mesangial cells (4). In addition, some of these binding sites were visualized by polyclonal antibodies raised against 90-kDa (p90) and 60-kDa (p60) rat liver membrane AGE-binding proteins (12), suggesting that these receptors belong to a family of AGE receptors that share structural characteristics among different cell types and are well conserved among species.

The AGE-induced increase in collagen type IV mRNA was inhibited by antibodies to both these receptor proteins. Thus, mouse mesangial cells express AGE receptors, which appear capable of mediating the observed AGE-induced ECM mRNA changes (4).

That a significant change in ECM mRNA levels or collagen release required 72-hr incubation with AGEs suggests that the AGE effect was indirect. AGE proteins are known stimulators of tumor necrosis factor, interleukin 1, PDGF, and insulin-like growth factor 1 for monocyte/macrophages (21–23). All of these modulators have been shown to be synthesized by mesangial cells in vitro (24). The AGE-induced increase in collagen type IV mRNA was abrogated by an antibody to PDGF, suggesting that this growth factor may contribute to increased matrix synthesis by mesangial cells under conditions of augmented AGE deposition within the glomerulus—e.g., in chronic diabetes.

Correlation between in vivo and in vitro events is often confounded by the phenotypic alterations that many cell types undergo in culture. The ECM phenotype expressed by the mesangial cells used in this study resembled that occurring in vivo (6). The major collagen phenotype at the transcriptional and secretory levels was type IV collagen, whereas collagen type I was not detected. Therefore, the AGE-induced changes seen may be directly relevant to glomerular pathology in vivo.

Hyperglycemia may have effects on the glomerulus independent of AGE formation. An increase in mRNA level and intact protein secretion of fibronectin, laminin, and collagen type IV in rat mesangial cells cultured in medium with high glucose concentration was recently reported (25). Although this rapid up-regulation, which occurred 3 days after exposure to high glucose, argues against a singular role for AGEs, the underlying mechanism for this phenomenon has not been elucidated.

Despite long-term hyperglycemia and elevated AGEs levels, only 30–40% of diabetic patients develop diabetic nephropathy (3). This fact suggests that although hyperglycemia is an essential prerequisite for the development of
diabetic nephropathy, only certain susceptible individuals succumb to the metabolic abnormalities of chronic diabetes. Recent animal and epidemiological data suggest that the predisposition, or resistance, to the development of glomerulosclerosis is genetically determined (26). The mesangial cell line used in this study was derived from SJL/C57 mice, a strain that develops glomerulosclerosis when made transgenic for bovine growth hormone or simian virus 40 T antigen (27, 28). Therefore, these animals represent a suitable strain for the investigation of the role of metabolic stimuli in inducing glomerulosclerosis.

AGE products have recently been reported to accumulate in circulation and tissues of patients with renal failure (2). Therefore, it is possible that, as renal function deteriorates, the progressive AGE deposition within the kidney tissues could further aggravate matrix accumulation, accelerating the development of end-stage glomerulosclerosis. Because type IV collagen and the other ECM components analyzed herein are major constituents of diabetic glomerulosclerosis in vivo, our findings provide evidence that AGEs may be important in the development of diabetic nephropathy (29).

We thank Dr. H. K. Kleinman for providing the antibody for collagen type IV (HK68). This work was supported, in part, by National Institutes of Health Grant AG-6943 and AG-8245 to H.V.