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Advanced glycation end products up-regulate gene expression found in diabetic glomerular disease

(diabetes mellitus/competitive PCR/extracellular matrix/growth factor/aminoguanidine)

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ABSTRACT **Several lines of evidence suggest that the excessive accumulation of extracellular matrix in the glomeruli of diabetic kidneys may be due to reactive intermediates forming between glucose and matrix proteins called advanced glycation end products (AGEs). Normal mice received AGE-modified mouse serum albumin i.p. for 4 weeks, and glomerular extracellular matrix, growth factor mRNA levels, and morphology were examined. We found that AGE induced an increase in glomerular extracellular matrix α1(IV) collagen, laminin B1, and transforming growth factor β_1 mRNA levels, as measured by competitive PCR, as well as glomerular hypertrophy. The AGE response was specific because the coadministration of an AGE inhibitor, aminoguanidine, reduced all these changes. We conclude that AGEs affected expression of genes implicated in diabetic kidney disease and may play a major role in nephropathy.**

Diabetic nephropathy is the single most common cause of end-stage renal disease (1), with an annual cost of $3 billion in the United States, and diabetics reaching dialysis have a 2-fold excess mortality risk. The pathogenesis of the renal lesions, characterized by an excess of glomerular extracellular matrix (ECM) (2, 3), is still the subject of considerable controversy. Hyperglycemia plays a role in the nephropathy because activation of glomerular ECM genes and renal lesions were decreased or prevented by insulin in streptozotocin-treated rats (4). In addition, proteinuria was decreased by strict glycemic control in two recent clinical trials on insulin-dependent diabetics (5, 6). Although elevated glucose levels led to the up-regulation of type IV collagen, laminin B1, and fibronectin gene expression by mesangial and endothelial cells *in vitro* (7, 8), numerous studies have indicated that the effects of glucose may be, in part, indirect due to the formation of advanced glycation end products (AGEs), resulting from prolonged exposure of proteins to glucose. AGEs represent a heterologous group of compounds, deriving from the Amadori product, an early intermediate of the nonenzymatic reaction of glucose with free amino groups of proteins (9, 10). Excessive accumulation of AGEs has been implicated in diabetic complications (11) based on the diverse biological properties that include protein cross-linking, cellular activation, growth promotion, and vascular dysfunction (9, 10). For instance, arterial-wall AGE levels were elevated in diabetics, especially those with end-stage renal disease (12, 13). It has been difficult to separate the effects of hyperglycemia from those of AGEs. This distinction has been facilitated by the discovery of inhibitors, such as aminoguanidine (nG), which react with Amadori-derived products to form stable compounds and do not progress to the formation of reactive AGEs, thus preventing AGE–protein cross-linking (14). Among other effects, nG was shown to reduce AGE content of aortic tissue in long-term-diabetic rats (15) and attenuate the glomerular lesions in diabetic rats (16), suggesting that AGEs participate in the development of these lesions. When the response(s) of cultured renal mesangial cells to AGE–albumin was examined, we found that the *in vitro* expression and secretion of several ECM components were up-regulated through surface receptors (17, 18). Furthermore, normal rats chronically injected with AGE developed vascular dysfunction and glomerular lesions (19, 20), which were markedly reduced in those cotreated with nG.

The current study was undertaken to determine the contribution of AGEs to diabetic kidney disease by examining the molecular events in glomeruli after AGE administration.

MATERIALS AND METHODS

Preparation of Advanced Glycosylation End Products. Albumin-derived AGEs were prepared by incubating mouse serum albumin (mSA) (Calbiochem) with 50 mM glucose 6-phosphate as described (21). Unmodified mSA was incubated under the same conditions without glucose 6-phosphate as controls. AGE–mSA and unmodified mSA were purified as described (18). Lipopolysaccharide content was <1 pg/ml by limulus amoebocyte lysate assay (QCL-1000 from BioWhittaker). AGE was quantified by an AGE-specific ELISA (21) (AGE–mSA, 154 units/mg; unmodified mSA, <0.4 units/mg).

Animals. Normal 10-week-old SJL female mice (20–25 g; The Jackson Laboratory) received daily i.p. injections of AGE–mSA (6 mg/day, $n = 6$) (AGE–mSA) or AGE–mSA (6 mg/day) followed immediately by i.p. injection of nG (10 mg/day, $n = 5$) (AGE + nG). Unmodified mSA-injected mice (6 mg/day, $n = 7$) (mSA) and age-matched noninjected mice (normal) ($n = 4$) served as controls. Mice were subjected to this protocol for 4 weeks.

Glomerular Microdissection and Reverse Transcription *in Situ*. After anesthesia with sodium pentobarbital (50 μg per g of body weight, i.p.), mice were killed by decapitation. The left kidneys were perfused with saline containing collagenase and RNase inhibitors (Boehringer Mannheim). Glomeruli were isolated by microdissection, permeabilized, and sonicated; cDNA was obtained after reverse transcription *in situ* as described (22, 23). The right kidneys were saved for measurement of AGE and histologic examination.

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Abbreviations: AGE, advanced glycation end product; mSA, mouse serum albumin; nG, aminoguanidine; SMA, smooth muscle α-actin; ECM, extracellular matrix; PDGF, platelet-derived growth factor; TGF-β_1, transforming growth factor β_1.

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Primer Construction and Competitive PCR Primers. The mouse α1(IV) collagen primers were as described (22); the size of the corresponding amplified product was 484 bp. The primer pair for laminin B1 (sense, 5′-CAA GCT TGA GAG GAA CGT GG-3′, and antisense, 5′-TTA CCT TGG TCA CCG AGC-3′) yielded a 443-bp PCR product. The transforming growth factor β_1 (TGF-β_1) primer pair (sense, 5′-ATA CAG GGC TTT CGA TTC AGC-3′, and antisense, 5′-GTC CAG GCT CCA AAT ATA GG-3′) yielded a 360-bp PCR product. The platelet-derived growth factor B (PDGF-B) primer pair (sense, 5′-TGT GAG ACA ATA GTG ACC-3′, and antisense, 5′-TTG GAG TCA AGA GAA GCC-3′) yielded a 435-bp PCR product. The smooth muscle α-actin (SMA) primer pair (sense, 5′-CCT GAC GGA CTA CCT CAT G-3′, and antisense, 5′-CAA ATC AAA GCT TTG GGC AG-3′) yielded a 434-bp PCR product. The β-actin primer pair (sense, 5′-TCT AGG CAC CAA GGT GTG-3′) and antisense, 5′-TCA TGA GGT AGT CCG TCA GG-3′) yielded a 460-bp PCR product. α1(I) collagen primer pair (sense, 5′-TGG TCC CTC TGG AAA TGC TGG ACC-3′, and antisense, 5′-CAG GAG AAC CAG GAG AAC CAG G-3′) yielded a 297-bp PCR product as described (23).

These primers were chosen so that only specific mRNA-derived cDNA sequences, but not genomic DNA sequences, would be amplified. Restriction enzyme analysis was done on each of the PCR products to confirm the amplification of specific mRNA sequences.

Competitive PCR Assay. To quantitate cDNAs, competitive PCR assays were done by adding decreased amounts of mutant templates to glomerular cDNA (Fig. 1). The test template for all PCR reactions was an aliquot of cDNA collected from a pool of 50 glomeruli. Each aliquot corresponded to 1/10th of a glomerulus. The mutated cDNA templates competed with test cDNA on an equimolar basis as described (22–24). For α1(IV) collagen, a point mutation constructed to yield a new *Bcl* I restriction site resulted in two fragments of 219 and 265 bp. For laminin B1, TGF-β_1, PDGF-B, SMA, and β-actins, deletion cDNA mutant templates were developed to create 57-, 58-, 55-, 84-, and 103-bp deletions in the middle of the molecules, resulting in mutant cDNAs of 386, 282, 380, 350, and 357 bp, respectively.

After agarose gel electrophoresis, amplification bands stained by ethidium bromide were quantitated from the film negative by scanning laser densitometry (PDI Imageware Systems, Huntington Station, NY). As reported, the ratio of mutant to wild-type band density was calculated for each lane and plotted as a function of the amount of initial mutant template added to the reaction. The amount of glomerular cDNA was derived from linear regression analysis with duplicate or triplicate assays (22–24).

Kidney AGE Determination. Kidneys were homogenized, delipidated, and digested with collagenase as described (19). Kidney AGE was measured by ELISA, and collagen content was determined as described (19). Kidney AGE in units/mg of hydroxyproline was expressed as percentage (± SEM) change compared with mSA mice.

Light and Immunofluorescence Microscopy. Light and immunofluorescence microscopy were done as described (25). For immunofluorescence microscopy, frozen or paraffin-embedded kidney sections were used. Paraffin sections were deparaffinized and lightly trypsinized at 37°C. Sections were coated with rabbit anti-bovine RNase AGE (21), followed by biotin-conjugated goat anti-rabbit IgG (Tago) or biotin-conjugated goat anti-mouse IgG, IgM (Tago), and streptavidin-conjugated fluorescein isothiocyanate (Zymed). The sections were examined and coded; the fluorescence intensity was then graded on a 0 to 4+ scale as described (25).

Glomerular Volume. The profile areas of 100 glomeruli were measured with a computer-assisted planimeter for morphometric analysis as described (25). The mean glomerular volume was derived from the harmonic mean of the glomerular equatorial surface area (25).

Mean Glomerular Cell Number. The mean glomerular cell number was determined as described (26). Briefly, the nuclei of 50 successive glomerular profiles were counted by scanning hematoxylin/eosin-stained tissue sections in a serpentine fashion. Taking into account the mean cell number per glomerular profile (*C*), the simple mean of the glomerular equatorial area (*A*), and glomerular volume (*GV*), the mean glomerular cell number (*N*) was derived by the following equation: $N = (C/A) \times GV$.

The mean value of glomerular cell number derived from each kidney in each group of mice was expressed as a percentage of the mean value derived from those in mSA mice.

Statistical Methods. Differences between groups were analyzed with the unpaired Student's *t* test. All values are expressed as means ± SEMs.

RESULTS

Quantitation of Glomerular mRNAs. The amount of cDNA per glomerulus quantitated in the attomole range was ex-

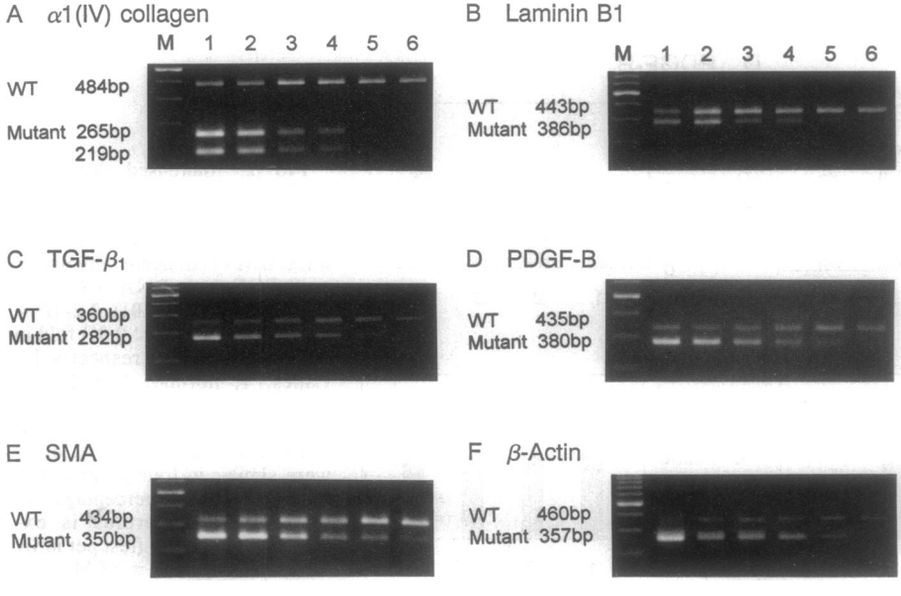

FIG. 1. Competitive PCR for glomerular α1(IV) collagen, laminin B1, TGF-β_1, PDGF-B, SMA, and β-actin cDNA. The template cDNAs for PCR reactions were mRNA reverse-transcribed *in situ* from pooled microdissected glomeruli. Decreased amounts of mutant templates were added to tubes containing test wild-type cDNA (WT) (lanes 1–6). Mutants were constructed by creating new restriction sites [α1(IV) collagen] or deletions (laminin B1, TGF-β_1, PDGF-B, SMA, β-actin) that were separated from PCR products by agarose gel electrophoresis. M, 100-bp marker.

Table 1. Glomerular mRNA quantitation by competitive PCR

	n	α1(IV) collagen	Laminin B1	TGF-β1	PDGF-B	SMA	β-Actin
Normal	4	111.6 ± 22	51.9 ± 1.1	30.0 ± 6.6	6.7 ± 1.0	124.0 ± 14.3	245.3 ± 24
mSA	7	115.6 ± 20.4	48.5 ± 6.1	28.4 ± 2.6	8.0 ± 0.7	129.3 ± 20.4	218.5 ± 35.4
AGE–mSA	6	198.3 ± 16.8*	107.5 ± 16.8*	43.2 ± 5.1†	8.4 ± 0.7	121.3 ± 19.8	182.2 ± 34.8
AGE + nG	5	157.8 ± 22.9	47.2 ± 5.2‡	32.9 ± 7.9	9.8 ± 1.0	88.0 ± 20.8	224.0 ± 59.6

Data are expressed as mean (amol per glomerulus × 10^{-4}) ± SEM from duplicate or triplicate assays.
*$P < 0.01$, AGE–mSA vs. mSA or normal.
†$P < 0.05$, AGE–mSA vs. mSA or normal.
‡$P < 0.01$, AGE + nG vs. Age–mSA.

pressed as amol per glomerulus × 10^{-4} (Table 1). The relative amount (percentage) of cDNA per glomerulus was expressed by the ratio test per mSA mice × 100 (mean ± SEM) (Fig. 2). α1(IV) collagen mRNA was increased 1.7-fold in AGE–mSA mice (normal, 96.5 ± 19%; mSA, 100.0 ± 17.7%; AGE–mSA, 171.5 ± 14.5%) (AGE–mSA vs. mSA or normal, $P < 0.01$). The α1(IV) collagen mRNA levels in AGE + nG mice (141.4 ± 20.5%) were not significantly different from mSA or normal mice. Laminin B1 mRNA increased 2.2-fold in the AGE–mSA mice (normal, 107.0 ± 2.3%; mSA, 100.0 ± 12.7%; AGE–mSA, 221.6 ± 34.7%) (AGE–mSA vs. mSA or normal, $P < 0.01$). nG abrogated the laminin B1 mRNA increase (AGE + nG, 97.3 ± 10.8%) (AGE + nG vs. AGE–mSA, $P < 0.01$). TGF-β1 mRNA was increased 1.5-fold in AGE–mSA mice (normal, 105.5 ± 12.2%; mSA, 100.0 ± 7.8%; AGE–mSA, 152 ± 18.1%) (AGE–mSA vs. mSA or normal, $P < 0.05$). The TGF-β1 mRNA levels in AGE + nG mice (AGE + nG, 115.8 ± 27.8%) were not significantly different from mSA or normal. PDGF-B, SMA, and β-actin mRNA levels were similar in four groups.

Kidney Weight and Mean Glomerular Volume. The glomerular volume/body weight ratio increased 39% in AGE–mSA mice (normal, 95 ± 7.1%; mSA, 100 ± 1.9%; AGE–mSA, 139.3 ± 3.3%) (AGE–mSA vs. mSA or normal, $P < 0.001$) (Fig. 3). The increase in glomerular volume was largely abrogated in AGE + nG mice (AGE–mSA, 116.9 ± 5.4%) (AGE–mSA vs. AGE + nG, $P < 0.005$; AGE + nG vs. mSA, $P < 0.05$). The kidney weight/body weight ratios were similar in four groups. Data are expressed as percentage change of each group compared with mSA mice.

Measurement of Kidney AGE Levels. Kidney AGE levels were measured by ELISA in units/mg of hydroxyproline and are expressed as percentage change compared with mSA mice (Fig. 4). There was a significant increase of kidney AGE levels in AGE–mSA mice (normal, 115.6 ± 15.2%; mSA, 100 ± 7.6%; AGE–mSA, 152.2 ± 10.3%; AGE–mSA vs. mSA, $P < 0.001$; AGE–mSA vs. normal, $P < 0.05$). The kidney AGE level in AGE + nG mice was reduced (AGE + nG, 106.7 ± 12.5%; AGE–mSA vs. AGE + nG, $P < 0.01$).

Light and Immunofluorescence Microscopy. AGE was absent in the glomeruli of mSA mice and accumulated in the mesangium of AGE–mSA mice (mean score, 3) (Fig. 5). Reduced accumulation was found in AGE + nG mice (mean score, 1). There were traces of IgG and IgM in mesangial spaces both in AGE and AGE + nG mice. Apart from the increase in size, no obvious glomerular changes were detected by light microscopy in the AGE–mSA mice (data not shown).

Mean Glomerular Cell Number. There were no statistically significant differences of mean glomerular cell number among the four groups (98.2 ± 7.1% in normal mice; 100.0 ± 5.4% in mSA mice; 103.6 ± 6.1% in AGE–mSA mice; 108.2 ± 6.2% in AGE + nG).

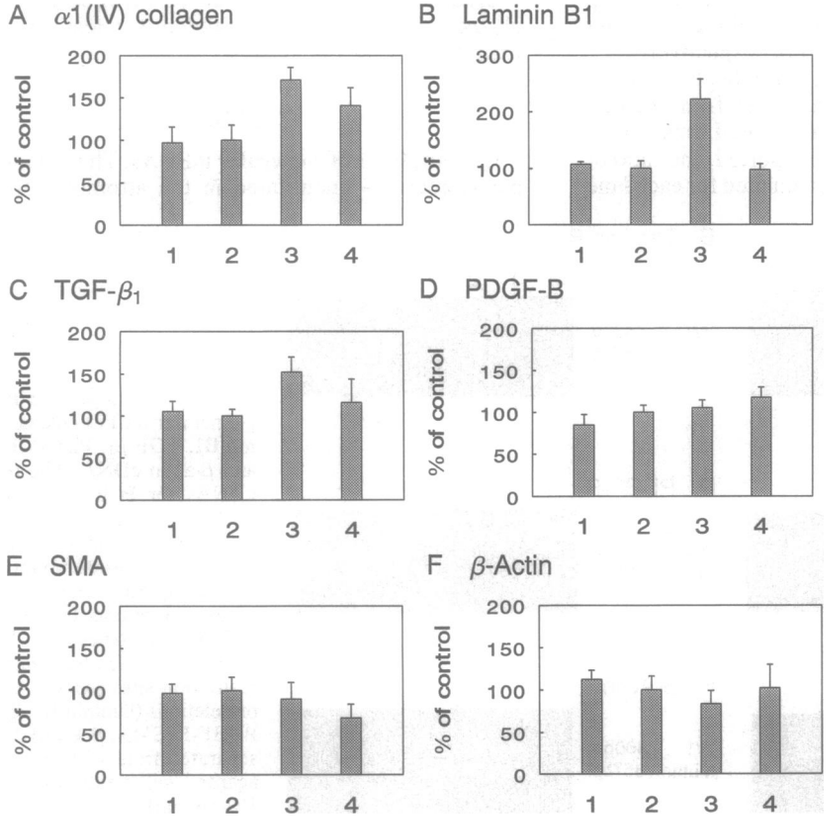

FIG. 2. Increased glomerular α1(IV) collagen, laminin B1, TGF-β1 but not PDGF-B, SMA, or β-actin mRNAs in AGE–mSA mice. α1(IV) collagen, laminin B_1, and TGF-β1 mRNA were increased 1.7- ($P < 0.01$), 2.2- ($P < 0.005$), and 1.5- ($P < 0.05$) fold in AGE–mSA mice, respectively. Lanes: 1, normal; 2, mSA; 3, AGE–mSA; 4, AGE + nG. nG reduced the increase. PDGF-B, SMA, and β-actin mRNA levels were similar in four groups. The relative amount (percentage) of cDNA per glomerulus is expressed by the ratio (test per mSA mice) × 100 (mean ± SEM).

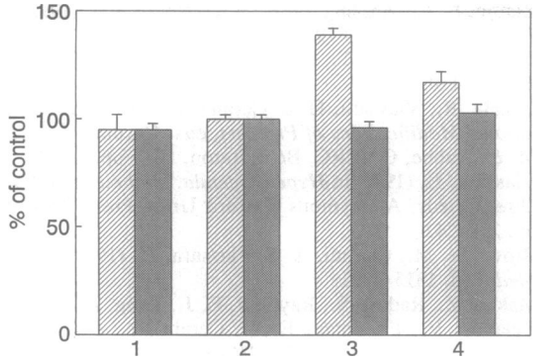

FIG. 3. Glomerular hypertrophy in AGE–mSA mice was reduced in mice cotreated with nG. The glomerular volume per body weight ratio (hatched bars) was increased 39% ($P < 0.001$) in AGE–mSA mice. Lanes: 1, normal; 2, mSA; 3, AGE–mSA; 4, AGE + nG. The increase in glomerular volume was largely abrogated in AGE + nG mice. The kidney weight/body weight ratios (solid bars) were similar in four groups. Data (± SEM) are expressed as percentage change of each group compared with mSA mice.

DISCUSSION

The *in vivo* administration of AGE induced a pattern of increased ECM gene expression similar to that found in experimental diabetic nephropathy. For instance, laminin B1 and type IV collagen mRNAs were increased in streptozotocin-treated diabetic rat glomeruli (4). In AGE–mSA mice, glomerular laminin B1, α1(IV) collagen mRNA were increased 2.2- and 1.7-fold, respectively. This up-regulation was not due to an increased cell number in the glomeruli. In contrast, these mRNAs did not differ in AGE + nG mice from uninjected or mSA mice.

AGE accumulation was readily detectable in the mesangial areas of AGE–mSA mice at 4 weeks by immunofluorescence microscopy, consistent with increased AGE associated with kidney collagen measured by an ELISA assay. However, AGE accumulation was markedly reduced in AGE + nG mice. Coupled with the consistent inhibition of AGE-induced changes throughout these experiments, these data lend credence to the postulate that there is a causal link between the presence of AGE moieties within the glomerular ECM and the observed responses. Although interstitial collagen (type I) has been observed in advanced human diabetic glomerular lesions (27), it was not found in this study, possibly due to the early phase of the lesions.

AGEs have been shown to mediate the secretion of several cytokines and growth factors, including PDGF and insulin-like growth factor IA through AGE-specific receptors on

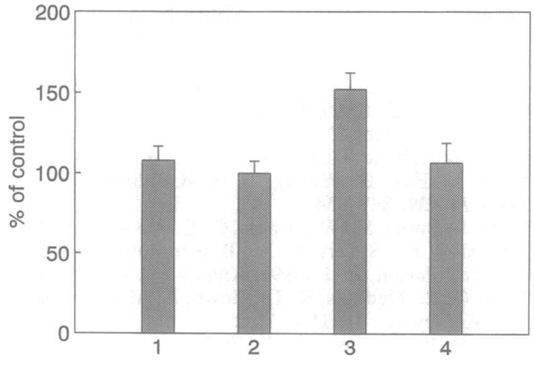

FIG. 4. Increased total kidney AGE levels in AGE–mSA mice. AGE was measured by ELISA (21) in units/mg of hydroxyproline and is expressed as percentage (± SEM) change compared with mSA mice. Lanes: 1, normal; 2, mSA; 3, AGE–mSA; 4, AGE + nG. nG reduced the increase.

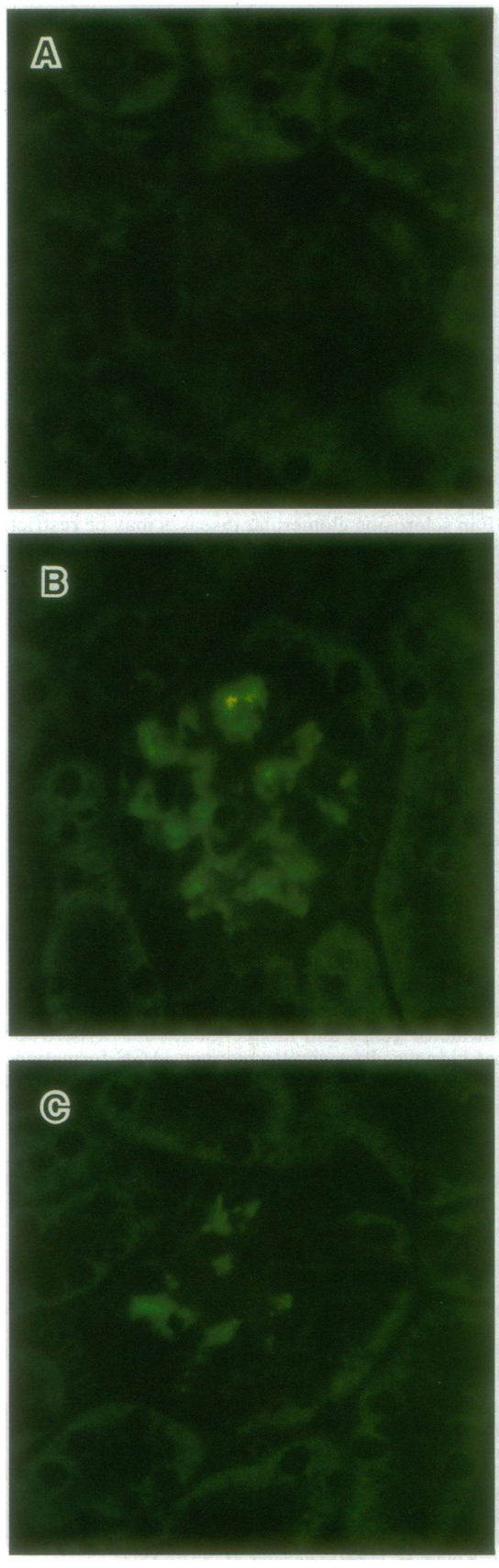

FIG. 5. The increased amount of AGE in the mesangial areas in AGE–mSA mice was reduced in AGE + nG mice. (*A*) AGE absence in the glomeruli of mSA mice. (*B*) Accumulation (+++) of AGE in the mesangium of AGE–mSA mice. (*C*) Reduced accumulation (+) of AGE in AGE + nG mice. (×380.)

monocyte/macrophages (28–31). In AGE–mSA mice, glomerular TGF-β_1 mRNA increased 1.5-fold, but PDGF-B mRNA did not increase. The induction of TGF-β_1 mRNA found here, as well as its suppression by nG, suggest that this peptide participates in the AGE response of the glomerulus *in vivo*. Interestingly, TGF-β_1 has been implicated in the increase of ECM in glomeruli of spontaneous nonobese diabetic mice (unpublished work), in experimental models of glomerular diseases, and in human diabetic glomeruli (32). TGF-β_1 also down-regulates the PDGF and PDGF-receptor autocrine system in some cells (33). Given the complex network of cytokines and growth factors controlling glomerular growth and matrix production (34), the AGE effects *in vivo* may not be mediated through local production of PDGF-B, although a role for exogenous PDGF cannot be excluded. Thus TGF-β_1 may mediate the changes in ECM gene expression noted here. This result may explain the fact that whereas in cultured mesangial cells the AGE-induced increase in collagen type IV mRNA was mediated, in part, through PDGF (18), PDGF-B mRNA was not increased in the glomeruli from AGE–mSA mice. Furthermore, the role of PDGF in sclerosing glomerular diseases may be restricted to those diseases with mesangial proliferation (35, 36), a feature not present in AGE–mSA mice. SMA, a marker of mesangial cell activation in glomerulonephritis models (37), was not increased in AGE–mSA mice, and β-actin mRNA levels were similar in all groups.

The glomerular hypertrophy, known to occur in insulin-dependent diabetic patients and in experimental animals (38), was also reproduced by AGE injection. In addition, this response was reduced by the coadministration of nG. The absence of concomitant overall kidney changes suggests that the glomeruli have a lower threshold of susceptibility to AGE-induced changes than other renal structures. This hypothesis is consistent with previous observations that total kidney and glomerular responses can be independently regulated (39, 40). The glomerular changes were observed at 4 weeks of AGE administration. Preliminary experiments revealed that glomerular volume or mRNAs were not changed after 7 days of AGE injections (data not shown).

The present study provides *in vivo* evidence that AGEs influence the expression of selected glomerular ECM and growth factor genes and induce glomerular hypertrophy in normal kidneys. In a similar manner, AGE formation may play a critical role in both the initiation and progression phases of human diabetic nephropathy. Finally, the present work suggests that AGEs mediate glomerular changes independently of other metabolic or genetic factors and may be blocked by agents such as nG. The studies provide a rationale for determining whether similar treatment might affect diabetic nephropathy in humans.

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