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Advanced glycation end products modulate transcriptional regulation in mesangial cells

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Advanced glycation end products modulate transcriptional regulation in mesangial cells. Advanced glycation end products (AGEs) stimulate synthesis of extracellular matrix (ECM) in a receptor-mediated manner on mesangial cells. In the present study, we examined the transcriptional regulation of the gene for type IV collagen [(IV)collagen], which is one of the major components of mesangial sclerosis, after stimulation of AGEs on mesangial cells. The methylation pattern of the promoter/enhancer region of (IV)collagen gene was similar in AGE-treated and control cells. AGEs significantly increased the transcriptional activity of the (IV)collagen gene, as measured by transient transfection assays using the reporter gene construct containing (IV)collagen promoter/enhancer and the chloramphenicol acetyltransferase gene. AGEs also increased smooth muscle α-actin mRNA levels as well as its transcriptional activity. Nuclear factor binding of the promoter of (IV)collagen gene was stimulated by AGEs. Furthermore, AGEs dramatically decreased the mRNA levels of (IV)collagen promoter binding protein (MSW), a larger subunit of DNA replication complex, AP1. These results suggest that AGEs increase expression of (IV)collagen gene by modulating the levels of promoter binding proteins. These transcriptional events may play a critical role in ECM accumulation in response to AGEs.

Advanced glycation end products (AGEs) are a complex mixture of adducts that have been recognized as a brown fluorescent material. They form cross-links between proteins by nonenzymatic reactions between glucose and amino groups of proteins. AGEs play an important role in biological and pathophysiological responses of disease and normal growth, including diabetic complications and the aging process [1, 2]. Since specific receptors for AGEs are found in various cells such as endothelial cells, macrophages, fibroblasts and mesangial cells, the responses to AGEs are thought to be mediated by specific receptors [3, 4]. These effects are mediated by some cytokines or growth factors (such as tumor necrosis factor α, interleukin-1, platelet-derived growth factor, insulin-like growth factor-1); however, the precise mechanism of the responses to AGEs is still not clear [5, 6].

In diabetic nephropathy, one of the major causes of end-stage kidney disease in Japan and the United States, there is accumulating evidence that AGEs have a pathogenic role in the development of diabetic glomerulopathy. The characteristic glomerular findings of diabetic nephropathy are both mesangial cell proliferation and accumulation of extracellular matrix (ECM) in mesangial area. The accumulated ECM includes type IV collagen [(IV)collagen], laminin, fibronectin and type I collagen [(I)collagen] [7]. We reported that AGEs bound to a specific receptor on mesangial cells and increased the synthesis of ECM. The AGEs responses were determined at the transcriptional level, however, factors controlling gene expression have not been determined.

One report about direct interaction between genes and AGEs indicated a direct structural modification of DNA as a result of complex formation [8]. Recent reports have also show that AGEs activate transcription factor NF-κB, which may stimulate the transcriptional activation of cytokines and cell adherence molecules [9]. These findings suggest a mechanism for the biological effects of oxidant stress; however, the transcriptional regulation of gene expression mediated by the other mechanism has not been elucidated. Since (IV)collagen is a major component of the expanded ECM in diabetic glomerulopathy, it is important to clarify the mechanisms by which the (IV)collagen is regulated at the transcriptional level in pathologic conditions. In this paper we focused on the effect of AGEs on the transcription of the (IV)collagen gene. We found that AGEs promote the transcription of the (IV)collagen gene by modulating several transcription factors. Recent reports suggest a phenotypic change of mesangial cells in glomerular injuries, reflecting an increase of smooth muscle α-actin (SMA) expression [10]. These phenotypic changes were also found in several human diseases including IgA nephropathy, diabetic nephropathy, membranoproliferative glomerulonephritis and focal glomerulosclerosis. However, the causal mechanisms are still unknown. We also showed that the SMA gene was up-regulated transcriptionally as a consequence of AGEs stimulation.

Methods

Cell culture

A glomerular mesangial cell line was established from glomeruli isolated from normal 4-week-old mice (C57BL/6J x SJL/J) and was identified according to a method described previously [3, 11]. We used phenotypically stable cells, passages 14 to 24, maintained in B medium and 20% fetal bovine serum (Irvine Scientific) and passaged weekly with trypsin-EDTA.

Preparation of ligands

AGE-bovine serum albumin (BSA) was prepared by the regular methods as described previously [3, 12, 13]. Unmodified BSA was

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incubated under the same conditions without glucose 6-phosphate for controls. Protein concentration was determined by the Bradford method using BSA as a standard. All AGE-protein specific fluorescence determinations were performed by measuring emission at 440 nm upon excitation at 370 nm using a fluorescence spectrometer (Hitachi). AGE content was estimated by fluorescence intensity at a protein concentration of 1 mg/ml. AGE-BSA contained 57.3 AGE units per mg of protein, and unmodified BSA contained 2.63 AGE units per mg of protein.

RNA isolation

Mesangial cells (1.2×10^6) were plated in medium/20% fetal bovine serum in 100 mm dishes coated with AGE-BSA or control BSA at 25 $\mu g/cm^2$ for 72 hours. Preliminary experiments revealed that the AGE-induced effect was not detectable up to 48 hours of incubation with AGEs. Therefore, subsequent experiments were done after 72 hours of exposure. The cells were washed with PBS, and total RNA was extracted by the guanidine isothiocyanate method [14].

RNA probe preparation

The cDNA (pl234) cloned in pGEM2 vector (Promega) codes for a portion of the major triple helical region plus the globular domain (NC1) of the mouse α1 (IV)collagen chain [15]. pGM101, pSAHE1, and pGMGAEH1 code for mouse α2 type I procollagen, mouse SMA, and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively [16, 17]. The cDNA insert [3.5 kilobase pairs (kbp)] for murine A1-p145 [MSW: (IV)collagen promoter binding protein] was subcloned into *Eco*RI site of Bluescript SK (-) (Stratagene) [18]. Orientation of the insert was determined by restriction enzyme digests.

Hybridization probes were prepared by linearizing the constructs with *Apa*I for pGM101, *Pvu*II for pl234, *Hin*dIII for pSAHE1, *Eco*RI for pGMGAEH1 and *Pvu*II for MSW. Before transcription *in vitro*, the linearized pGM101 was treated with Klenow DNA polymerase (Promega) to convert the 3′ protruding ends to blunt termini. The *in vitro* transcription reaction contained 1 μg of template DNA, [^{32}P]UTP (NEB), and the SP6 or T7 RNA polymerase (Promega) as described [17, 19]. The 1 kilobase (kb) ladder or 100 base pair (bp) ladder, which was labeled with [^{32}P]dCTP 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 Wako Pure Chemical Industries, Ltd.

Solution hybridization RNase protection assay

The RNA probe (4×10^5 cpm) and 1 μg of total RNA were hybridized overnight at 45°C. RNase A (40 μg/ml) was added to each tube and incubated for one hour at 30°C. RNase-resistant fragments were analyzed by 3.5% or 7% polyacrylamide/8 M urea gel electrophoresis and autoradiography [17]. The protected band of each RNA probe had the same size as the coding sequence for specific mRNA providing evidence for their specificity.

DNA transfection

The α1 (IV)collagen promoter-enhancer-CAT construct (p56) was described previously [20]. The construct SMP-1 contains 1074 bp of the proximal 5′-flanking region plus 43 bp 5′-untranslated region of the SMA gene that is cloned into the *Sal*I-*Bam*HI site of the SV40 enhancer containing CAT vector, pBLCAT3 [21]. Four $\times 10^5$ cells were plated on a 60 mm dish for 90 minutes and transfected with plasmid DNA using the standard calcium phosphate coprecipitation technique [22]. Typically, 15 μg of CAT reporter plasmid and 4 μg of pSV-β-galactosidase (Promega) (internal control for transfection efficiency) were used. The cells were exposed to the precipitates for four hours, washed and B medium containing 20% FBS was again added. After 48 hours, the cell layers were harvested and assayed for CAT and β-galactosidase activity [23, 24]. CAT (Pharmacia) or β-galactosidase (Promega) were used as standards. The pSV0CAT and pSV2CAT were used for negative and positive controls, respectively [22]. A correction factor for differences in transfection efficiency was obtained by normalizing CAT activity to the β-galactosidase activity and subtracting the background level, as determined by transfection with pSV0CAT. The final level was expressed as relative CAT activity.

DNA methylation pattern

High molecular weight cellular DNA was prepared from each cell line after 72 hours of incubation with AGE-BSA or control BSA, by the procedure of Britten, Graham and Neufeld [25]. Aliquots (15 μg) of DNA were digested with *Hpa*II or *Msp*I restriction endonucleases (5 units/μg of DNA) for 18 hours at 37°C. DNA samples were electrophoresed on a 1% agarose gel and transferred to Nytran by the Southern method [26]. Specific band patterns were detected by specific probes for the promoter and enhancer elements of the α1 (IV)collagen gene (*Xho*I fragment of p56) [20, 27], the α2 (I)collagen gene (*Xho*I fragment of pGM101) [28], or promoter elements of the SMA gene (*Hin*dIII-*Bam*HI fragment of the 5′ end SMP-1) [29]. These fragments were labeled by the random primer method [30].

Preparation of nuclear extract

Subconfluent cells incubated with AGE-BSA or control BSA for 72 hours were collected at 4°C and resuspended in hypotonic buffer (10 mM HEPES, pH7.9/1.5 mM $MgCl_2$/10 mM KCl/0.2 mM phenylmethylsulfonyl fluoride (PMSF)/0.5 mM dithiothreitol (DTT)) and allowed to swell on ice 10 minutes. The cells were homogenized in a Dounce homogenizer. The nuclei then were pelleted at 4°C and resuspended in low-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol/1.5 mM $MgCl_2$/20 mM KCl/0.2 mM EDTA/0.2 mM PMSF/0.2 mM DTT). An equal volume of high-salt buffer (20 mM HEPES, pH 7.9/25% glycerol/1.5 mM $MgCl_2$/1.2 M KCl/0.2 mM EDTA/0.2 mM PMSF/0.2 mM DTT) was added. The nuclei were centrifuged for 30 minutes at 25,000 $\times$ g. The nuclear extracts were dialyzed against 200 ml dialysis buffer (20 mM HEPES, pH 7.9/20% glycerol/100 mM KCl/0.2 mM EDTA/0.2 mM PMSF/0.5 mM DTT) overnight [31]. Protein concentration of nuclear extract was determined by the Bradford assay [32].

Gel mobility-shift assay

The following three oligonucleotide pairs were synthesized (Kurabo). CIV-1 (5′-TTCCTCCCCT TGGAGGAGCG CCGCCCG-3′) contains the binding site of A1-p145 from the promoter of α1 (IV)collagen gene. FT-A (5′-ACTCCTTATC TCTGATGGCG-3′) and FT-B (5′-CAGTGTCATA ACAGCCAGGG-3′) included a site for nuclear factor binding from the (IV)collagen enhancer region. They were end-labeled with [^{32}P]ATP by T4 polynucleotide kinase and purified by resin column filtration (Clontech). These oligonucleotides were incubated with 4 μg of

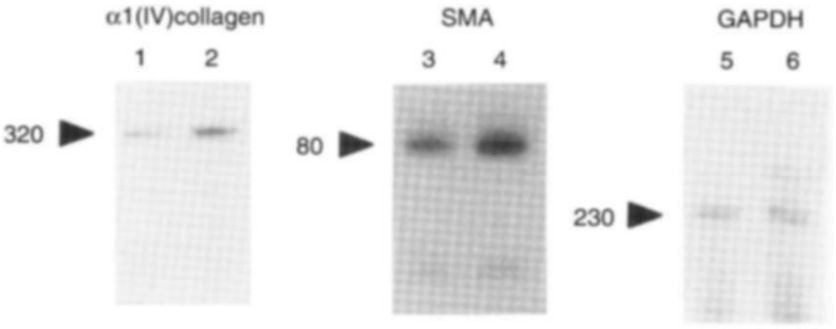

Fig. 1. *Effect of AGEs on mRNA synthesis in mesangial cells.* Levels of mRNAs coding for ECM were analyzed by solution hybridization protection assay. Cells were exposed to control BSA (lanes 1, 3, 5) or to AGE-BSA (lanes 2, 4, 6). α1 (IV)collagen (lanes 1, 2), SMA (lanes 3, 4), GAPDH (lanes 5, 6). There are many extra small bands in lanes 3 and 4. These small bands in the tRNA control lane can be detected without total RNA (data not shown).

nuclear extracts and 1.5 μg of poly (dI-dC) in a final volume of 10 μl containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 10% glycerol, and 0.05% nonidet P-40. Binding was allowed to proceed at room temperature for 20 minutes. The samples were analyzed by a 5% non-denaturing polyacrylamide gel electrophoresis in a low ionic strength buffer and autoradiography [33]. In gel supershift assay, 1.0 μl of appropriate diluted anti-MSW antibody or irrelevant antibody were added to the 10 μl of a standard gel shift reaction, after the addition of the ^{32}P-labeled oligonucleotide [18]. For the competition assay, Sp1 consensus oligonucleotide (5′-ATTCGATCGG GGCGGGGCGA GC-3′) (Santa Cruz Biotechnology, Inc.) was used.

Statistical analysis

Data were expressed as the mean ± SE and evaluated with analysis of non-parametric test (Mann-Whitney analysis).

Results

mRNA levels after stimulation with AGEs

The mRNAs for (IV)collagen, SMA and GAPDH were easily detectable in mesangial cells (Fig. 1), whereas (I)collagen mRNA was not detectable (data not shown). The mRNA levels for (IV)collagen and SMA were significantly increased after incubating cells with AGE-BSA for three days, compared with BSA alone. This difference was not detectable at day 1 and disappeared at seven days (data not shown). A GAPDH mRNA level was equivalent in cells plated on control and AGE-BSA.

Methylation state and regulation of transcription

To elucidate the mechanisms of transcriptional change, we examined the DNA methylation state in the transcriptional regulatory elements (cis-elements) of these genes with a Southern blot analysis. The methyl-sensitive restriction enzyme, *Hpa*II, did not cut CCGG sites if the internal cytosine was methylated, yet its isoschizomer, *Msp*I, did cut the internal cytosine methylated CCGG sites. Neither enzyme cut if the external cytosine was methylated [34].

The methylation pattern of the promoter elements of the SMA genes was examined in AGE-treated and control cells (Fig. 2A). Control cells showed specific bands of 10 and 1.3 kbp with *Hpa*II digestion and 4.3 and 1.3 kbp bands with *Msp*I digestion, which indicated that the promoter elements of the SMA gene were partially methylated. AGE-treated cells showed the same band patterns with *Hpa*II digests as the control cells. However, the *Msp*I digestion exhibited specific bands of 4.3, 1.6 and 1.3 kbp. The presence of a 1.6 kbp band indicated that AGEs induced demethylation of the external cytosine of CCGG in the promoter element.

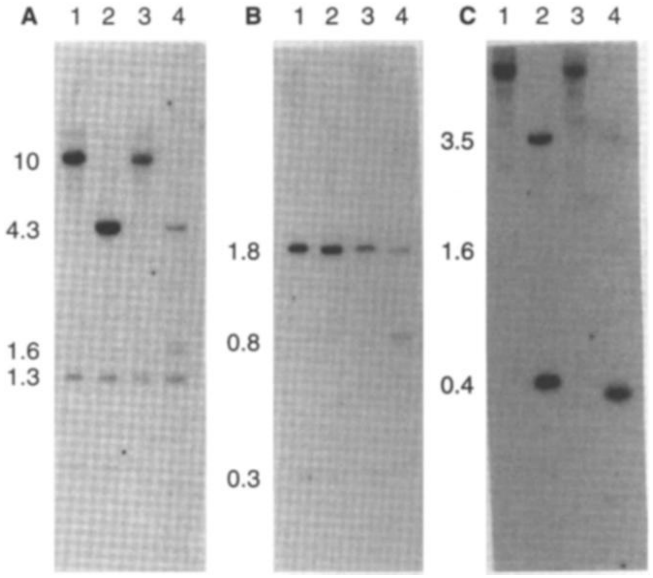

Fig. 2. *Methylation pattern of the regulatory elements of the (I)collagen, (IV)collagen and SMA genes with AGEs stimulation.* DNA was isolated from both AGEs stimulated mesangial cells and control cells. DNA was digested with *Hpa*II or *Msp*I, electrophoresed, and blot-hybridized with a probe to the regulatory elements of the (I)collagen gene, the (IV)collagen gene or the SMA gene as described in the **Methods** section. The size of the fragments is shown in kilobases. Mesangial cells stimulated by AGEs are represented by lanes 3 and 4, whereas control cells are represented by lanes 1 and 2. Lanes 1 and 3 were digested with *Hpa*II; lanes 2 and 4 were digested with *Msp*I. **A.** The promoter element of the SMA gene was used as a hybridization probe. The presence of specific bands of 10 and 1.3 kbp with *Hpa*II digestion, and 4.3 and 1.3 kbp bands with *Msp*I digestion, which indicated the gene was partially methylated. The presence of 1.6 kbp band in lane 4 indicated that AGEs induced demethylation of the external cytosine of CCGG in the promoter element. **B.** The promoter and enhancer elements of the (IV)collagen gene were used as a hybridization probe. The presence of 1.8 kbp and 0.3 kbp band in all lanes indicated that the internal cytosine of CCGG was unmethylated. The existence of 0.8 kbp band in lane 4 indicated that AGEs induced demethylation. **C.** The promoter and enhancer element of the α2 (I)collagen gene was used as a hybridization probe. The presence of 3.5 kbp and 0.4 kbp bands indicated the gene was methylated in both cells. The presence of 1.6 kbp band in lane 4 also indicated that AGEs induced demethylation.

The methylation patterns of the α1 (IV)collagen promoter and enhancer elements were evaluated with *Msp*I and *Hpa*II (Fig. 2B) [35]. The gene was unmethylated in both AGEs treated and

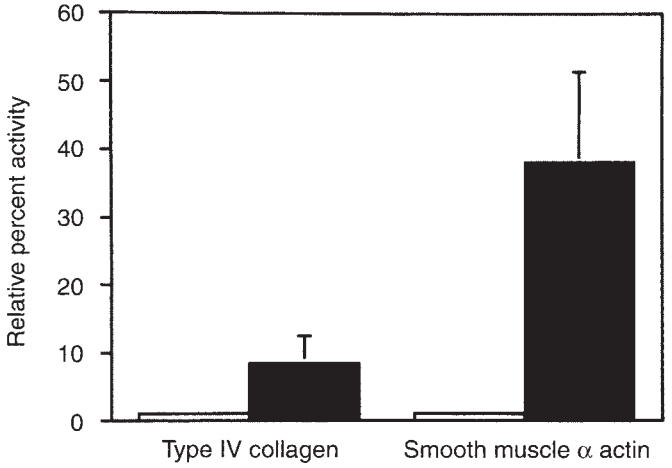

Fig. 3. *Effect of AGEs on transcriptional activity.* Transient transfection of mouse mesangial cells was performed as described in the **Methods** section, and chloramphenicol acetyltransferase (CAT) activity was determined 48 hours after transfection. The values shown are percentages of total chloramphenicol converted to acetylated derivatives. The pSV0CAT and pSV2CAT were used for negative and positive controls. This percentage was calculated from data obtained by counting scintillation cocktail including extracted labeled product and was corrected by transfection efficiency and background level. The results shown are the means ± SE of four separate experiments. Symbols are: (■) AGEs treated; (□) control.

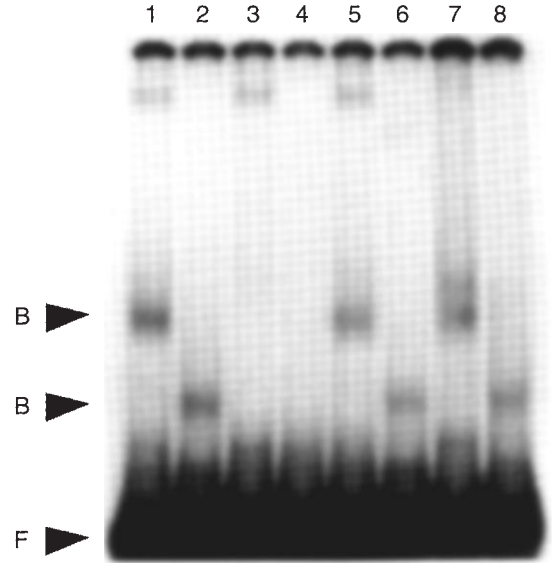

Fig. 4. *Binding of nuclear factors to the CIV-1 oligonucleotides.* Gel mobility shift assay of the (IV)collagen promoter with mouse mesangial cell nuclear extracts. The CIV-1 oligonucleotide from the promoter region was radioactively labeled and was incubated with nuclear extracts isolated from mouse mesangial cells (lanes 1 to 8). In lanes 2, 4, 6 and 8, the nuclear extracts were isolated from AGEs stimulated cells. Lanes 1, 3, 5 and 7, the nuclear extracts were isolated from control cells. Lanes 1 and 2, no competitor; lanes 3 and 4, reactions contained 100-fold molar excess of unlabeled oligonucleotides. Lanes 5 and 6, reactions contained 100-fold molar excess of SP1 consensus motif oligonucleotides. Lanes 7 and 8, reactions contained anti-MSW antibody. Super shifted band was detected in lane 7.

control cells, in agreement with its high level of expression. The *Msp*I digests showed a 1.8 kbp band as well as a 0.3 kbp band in these cells, indicating that the external cytosine was methylated. The existence of 0.8 kbp band with the *Msp*I digestion indicated that AGEs induced demethylation.

The methylation pattern of promoter/enhancer elements of the α2 (I)collagen gene was determined in AGE-treated and control cells (Fig. 2C). As judged by the presence of 3.5 kbp and 0.4 kbp bands in the *Msp*I I digests, the gene was methylated in both cells, thus the α2 (I)collagen gene was not expressed. The presence of 1.6 kbp band, in the *Msp*I digests of AGE-treated cells also indicated partial demethylation in promoter/enhancer elements of the α2 (I)collagen gene.

Trans-acting elements

The role of trans-acting elements in the regulation of transcription was investigated by transiently transfecting cells with fusion constructs containing the promoter/enhancer/chloramphenicol acetyltransferase (CAT) for each gene. Correction for transfection efficiency was accomplished by cotransfection with pSV-β-galactosidase as an internal control. pSV0CAT and pSV2CAT were used as negative (0%) and positive (100%) controls, respectively. AGEs treated cells or control cells were transfected with target constructs by the calcium-phosphate method.

Transcriptional activity of the promoter of the SMA gene was analyzed by using the construct, SMP-1, which contained the SMA promoter, the SV40 enhancer and the CAT reporter gene (Fig. 3). CAT activity was increased 37-fold in AGE-treated cells compared with control cells, indicating that AGEs significantly stimulated promoter activities of the SMA gene. There was a correlation between the relative CAT activity and the levels of endogenous smooth muscle α-actin mRNA expression.

A construct P56, containing the promoter/enhancer of the α1 (IV)collagen gene and the CAT gene, was used for evaluation of transcriptional activity of (IV)collagen. AGEs induced a 16-fold increase in CAT activity compared with control, demonstrating that AGEs enhanced transcriptional activity of the α1 (IV)collagen gene (Fig. 3).

These data suggest that increased mRNA levels for SMA and (IV)collagen by AGEs are due to the increase in promoter activity of their genes. The promoter activity of SMA gene is regulated by both the methylation state of the enhancer and/or promoter elements and trans-acting elements. The promoter activity of α1 (IV)collagen gene is mainly regulated by trans-acting elements.

Regulation of transcription factors for the α1 (IV)collagen gene by AGEs

To further investigate the effect of AGEs on the transcriptional regulation of the α1 (IV)collagen gene, DNA binding proteins for the promoter and enhancer elements of the α1 (IV)collagen gene were examined by a gel mobility-shift assay.

Figure 4 shows the gel mobility shift of the CIV-1 site [a part of promoter sequence of the (IV)collagen genes] when incubated with nuclear extracts prepared from mesangial cells treated with AGE-BSA or with control BSA. The migration of the labeled CIV-1 site indicated an interaction between proteins and DNA. The intensity of the shifted band was dependent on the amounts of nuclear extracts (0.5, 1.5, 4.5 μg) (data not shown). The shifted band competed with the excess amounts of the unlabeled CIV-1 site DNA. Since the CIV-1 site contained a consensus sequence for Sp1 binding, the gel shift assay was performed with the Sp1

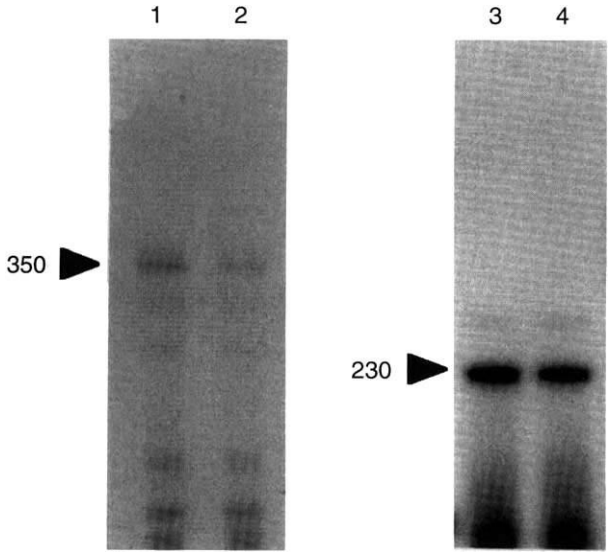

Fig. 5. *Effect of AGEs on MSW mRNA production.* mRNAs coding for MSW were measured by solution hybridization protection assay. Cells were exposed to control BSA (lane 1) or to AGE-BSA (lane 2). The result of densitometric analysis was 12.8 (lane 1) and 0.8 (lane 2). Densitometry readings were normalized for equivalent amounts of GAPDH RNA per sample to confirm equal loading of RNA.

binding sequence as an unlabeled excess competitor to rule out an involvement of Sp1. The shifted band then did not compete with the Sp1 consensus motif (Fig. 4). Control nuclear extracts showed one shifted band of the CIV-1 site. The CIV-1 site contained a binding sequence for MSW [(IV)collagen promoter binding protein]. Therefore, we performed gel supershift assays with anti-MSW antibody added to the standard gel shift reaction, and a supershifted band was detected in the control lane. On the other hand, AGE induced a differently sized shifted band that was not supershifted by adding anti-MSW antibody. Next the MSW mRNA levels were examined in these cells. Like the data from gel shift assays, the MSW mRNA levels were significantly decreased in AGE-treated cells (1/15.8-fold; Fig. 5). The results suggest that AGE decreased MSW and induced a novel DNA-binding protein of the CIV-1 site.

The labeled FT-A and FT-B sites from the enhancer region of the α1 (IV)collagen gene were also tested for their activity of nuclear factor binding. There was no difference between AGE-treatment and control (data not shown). These experimental results indicate that AGEs modulate some nuclear factors that bind to the promoter elements of the (IV)collagen gene.

Discussion

Significant advances in the understanding of advanced glycation end products (AGEs) for development of diabetic complications have been made in the past few years [1, 2]. Especially in diabetic nephropathy, AGEs accumulate in the glomeruli in nonobese diabetic mice [36] and diabetic humans [37]. The prescribed AGEs increased the glomerular extracellular matrix in the normal mouse [38]. Our previous report showed that AGEs increased synthesis of extracellular matrix (ECM) in mesangial cells through a specific receptor [3]. In this study, we examined the effect of AGEs on the transcriptional regulation for the (IV)collagen gene. Our findings suggest that this effect was mediated through multiple steps, including the increased nuclear factor binding of the promoter of the (IV)collagen gene accompanying with demethylation of these elements. These regulatory mechanisms induced by AGEs appear to be associated with the development of glomerulosclerosis. Our study shows that AGEs stimulate the synthesis of smooth muscle α actin (SMA), which is a marker for phenotypic change in the course of developing glomerulosclerosis [10]. These findings may provide new information about the pathogenic role of AGEs in the development of diabetic nephropathy.

The present study showed that CCGG in regulatory elements for the (I)collagen was highly methylated in associated with low gene expression in both control and AGE-treated cells. On the contrary, regulatory elements for the (IV)collagen gene and the SMA gene were not methylated in control and AGE-treated cells, which was consistent with high levels of gene expression. We also found that AGEs induced a demethylation in external cytosine of CCGG in all genes. However, the gene expression was not only regulated by demethylation in external cytosine, since AGEs induced demethylation of the regulatory element of the (I)collagen gene without mRNA expression. These findings were corroborated by evidence that the level of expression of the (I)collagen gene was independent of methylation [39]. A recent report showed that free radical adducts play a role in DNA cytosine demethylation [40], suggesting that demethylation of AGEs was mediated by formation of free radicals. This phenomenon suggests the possibility that AGEs may activate several genes and affect the differentiation state of the cells. On the other hand, AGEs directly form AGE-DNA and increase mutations [8]. These unexpected activities could account for the development of fetal abnormalities resulting from diabetic pregnancies or age-related cancers.

Transient transfection assays revealed that AGEs enhance transcriptional activity of both the (IV)collagen gene and the SMA gene, indicating the important role of trans-acting factors. The (IV)collagen gene consists of α1 chain and α2 chain genes in a head-to-head manner and regulated by 130 bp bi-directional promoter between the two genes and an enhancer in the first intron of the α1 (IV)collagen gene [27]. Recently a protein factor, MSW, which binds to the promoter, was cloned and sequenced [18]. MSW contains several domains homologous the smaller subunits of replication factor complex, A1, and appears to identical to a larger subunit, A145, of A1. Therefore MSW could play a role in both transcription and replication. This paper shows that AGEs induce some nuclear factor binding of the promoter of the (IV)collagen genes. The data from supershift assays using anti-MSW antibody shows that the promoter binding protein increased by AGEs is different from MSW. On the contrary, MSW itself is decreased after treatment with AGEs. The MSW-binding oligonucleotide (CIV-1) includes the Sp1 consensus sequences. The increased DNA binding protein is not Sp1, since it does not compete with excess Sp1 consensus sequences. No other known transcription factors binding this oligonucleotide were identified through a search of the Transcription Factor Database (version 7, National Center of Biological Information). These findings suggest that both down-regulation of MSW and up-regulation of novel promoter binding proteins are important for augmenting (IV)collagen gene expression by AGEs.

SMA is the most abundant protein in differentiated smooth muscle cells (SMC) and is one of the earliest proteins expressed

during the differentiation of SMC. Although SMA is expressed by a variety of mesoderm-derived cells during development, its expression in differentiated cells is limited to SMC and SMC-related cells. Thus we can use SMA as a phenotypic marker of mesangial cells [41]. The mechanism of the gene expression of SMA is partially revealed. In rat vascular smooth muscle cells, arginine vasopressin induced the gene expression through the CArG elements in the promoter of the SMA gene [42]. In mesangial injuries, the gene expression of SMA was increased in association with the mesangial cell phenotypic change. AGE stimulation also increased the gene expression of SMA as a consequence of transcriptional activation. It is important to find the transcription factors and the crucial regulatory elements involved in the SMA gene expression, and to identify the factor determining the phenotypic change.

An epidemiological study showed that only 30 to 40% of diabetic patients develop diabetic nephropathy. Recent animal and human data suggest that the predisposition, or resistance, to the development of glomerulosclerosis was genetically determined [43]. However, long-term hyperglycemia and elevated AGE levels were strongly implicated in the initiation and acceleration of multiple organ injuries in diabetes, including vasculature, kidneys, and nerves. This hypothesis is supported by the following facts: a recent sensitive immunohistochemical technique revealed that AGEs accumulated in the circulation and organ tissues of diabetic patients or aged people [44]; treatment for eight months with aminoguanidine, an inhibitor of AGE formation, exhibited inhibitory effects on glomerular lesions and retardation of albuminuria in diabetic model rats [45]; and there were scavenger systems for AGEs in several organs [46]. In this study, we clarified some unique regulatory mechanisms of AGEs for a pathological role in diabetic glomerulosclerosis.

Acknowledgments

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Appendix

The abbreviations in this paper are: AGEs, advanced glycation end products; (IV)collagen, type IV collagen; (I)collagen, type I collagen; ECM, extracellular matrix; SMC, smooth muscle cells; SMA, smooth muscle α-actin; BSA, bovine serum albumin; PBS, phosphate buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kbp, kilobase pairs; MSW, mouse southwestern; bp, base pair; CAT, chloramphenicol acetyltransferase; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol.

Reprint requests to Toshio Doi, M.D., Division of Artificial Kidneys, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

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