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Role of Receptor for Advanced Glycation End-Product (RAGE) and the JAK/STAT-Signaling Pathway in AGE-Induced Collagen Production in NRK-49F Cells

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Abstract Advanced glycation end-product (AGE) is important in the pathogenesis of diabetic nephropathy (DN), and captopril (an angiotensin converting enzyme inhibitor) is effective in treating this disorder. We have shown that the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) cascade is responsible for AGE-induced mitogenesis in NRK-49F (normal rat kidney fibroblast) cells, but its role in renal fibrosis in DN remains unknown. Therefore, we have sought to determine whether JAK/STAT is involved in AGE-regulated collagen production in NRK-49F cells. We found that AGE time (1–7 days) and dose (10–200 μg/ml)-dependently increased collagen production in these cells. Additionally, AGE increased RAGE (receptor for AGE) protein expression. AGE-induced RAGE expression was dose-dependently inhibited by antisense RAGE oligodeoxynucleotide (ODN) and captopril. AGE-induced type I collagen production and JAK2-STAT1/STAT3 activation were decreased by AG-490 (a specific JAK2 inhibitor), antisense RAGE ODN and captopril. Meanwhile, STAT1 and STAT3 decoy ODNs also suppressed the induction of collagen by AGE. We concluded that RAGE and the JAK2-STAT1/STAT3 pathway were involved in AGE-induced collagen production in NRK-49F cells. Furthermore, captopril was found to reverse AGE-induced collagen production, probably by attenuating RAGE expression and JAK2-STAT1/STAT3 activities. J. Cell. Biochem. 81:102–113, 2001.
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Key words: diabetic nephropathy; advanced glycation end-product; oligodeoxynucleotide; tyrosine phosphorylation; captopril

Diabetic nephropathy (DN) is characterized by an expansion of extracellular matrix (ECM), which results in renal fibrosis and end-stage renal disease [Lapuz, 1997]. In this regard, interstitial fibroblast is critical in renal fibrosis [Eddy, 1996]. In fact, tubulointerstitial damage may even be more important than glomerulopathy in terms of renal prognosis in DN [Ziyadeh and Goldfarb, 1991]. Therefore, we have been studying the mechanisms of tubulointerstitial damage in models of DN in vitro [Guh et al., 1996; Yang et al., 1998; Huang et al., 1999].

Additionally, increased intrarenal angiotensin II has been implicated in the pathogenesis

Abbreviations used: JAK, Janus kinase; STAT, signal transducers and activators of transcription; AGE, advanced glycation end-product; RAGE, receptor for AGE; ACEI, angiotensin converting enzyme inhibitor; BSA, bovine serum albumin; NRK-49F; normal rat kidney fibroblast; DN, diabetic nephropathy; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; ODN, oligodeoxynucleotide; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; DMSO, dimethylsulfoxide.

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of diabetic nephropathy [Wolf and Ziyadeh, 1997; Price et al., 1999]. For example, angiotensin converting enzyme inhibitor (ACEI, e.g., captopril), which inhibits angiotensin II, is effective in treating DN [Lewis et al., 1993; Kshirsagar et al., 2000] and preventing accumulation of ECM in the diabetic kidney [Gilbert et al., 1998].

Hyperglycemia is important in the pathogenesis of DN [Friedman, 1999]. Moreover, the non-enzymatic glycosylation between glucose and primary amino groups on proteins (Maillard reaction) forms Schiff bases, which rearrange into the Amadori products. Amadori products are then transformed to stable covalent advanced glycation end-products (AGE) [Friedman, 1999]. AGE has been shown to induce glomerular collagen expression in vivo [Yang et al., 1994] and inducing collagen in vitro in mesangial cells [Throckmorton et al., 1995], lens epithelial cells [Hong et al., 2000], and vascular smooth muscle cells [Mizutani et al., 2000].

Additionally, AGE accumulates in the kidney in various DN models [Friedman, 1999]. AGE binds to cell surface receptors, including receptors for AGE (RAGE), before exerting its effects [Schmidt et al., 1996]. RAGE is a member of the immunoglobulin superfamily expressed on many cells [Schmidt et al., 1996]. In sum, the interaction between AGE and RAGE has been postulated to be important in diabetic complications, including DN [Friedman 1999; Yamamoto et al., 2000].

The signaling pathways of AGE through its receptors, however, are not well defined. In this regard, AGE has been shown to activate oxidant stress and NF-κB in endothelial cells, and the mitogen-activated protein kinase (MAPK) pathway in renal tubular cells, respectively [Schmidt et al., 1996; Simm et al., 1997]. Moreover, we have previously shown that the JAK (Janus kinase)/STAT (signal transducers and activators of transcription) pathway is necessary for AGE-induced cellular proliferation in NRK-49F (normal rat kidney interstitial fibroblast) cells [Huang et al., 1999]. Interestingly, MAPK is required for the maximal activation of STAT [Wen et al., 1995]. Thus, there may be important interactions between various signaling pathways.

The JAK/STAT pathway transduces signals initiated by many agonists [Darnell, 1997; Ransohoff, 1998]. To date, there are four JAKs (JAK1, JAK2, JAK3, and TYK2) and seven STATs (STAT 1 to 4, 5a, 5b, and 6). STATs are tyrosine-phosphorylated by the activated JAKs and then translocated to the nucleus to become the transcription factors. Because collagen is the major tubulointerstitial ECM protein in DN [Gilbert et al., 1998] and fibroblast proliferation may be associated with an enhanced production of collagen [Makela et al., 1990], we speculate that the JAK/STAT pathway may also be involved in AGE-induced collagen production.

Therefore, we studied NRK-49F cells cultured in AGE-albumin to determine (1) if AGE affects collagen production in these cells, (2) if RAGE is involved in AGE-induced JAK/STAT cascade, (3) if RAGE and JAK/STAT are involved in AGE-induced collagen production, (4) what the effects of captopril are on AGE-activated JAK/STAT cascade and collagen production.

MATERIALS AND METHODS

Reagents

Fetal calf serum (FCS), DMEM, antibiotics, lipofectin, molecular weight standards, trypsin-EDTA, trypan blue stain, and all medium additives were obtained from Life Technologies, Inc. (Rockville, MD). Anti-phosphotyrosine (PY20), -RAGE, -JAK2, -STAT1, and -STAT3 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Protein A/G-coupled agarose beads, and tyrphostin AG-490 were purchased from Calbiochem Corp. (San Diego, CA). HRP-conjugated goat anti-rabbit or anti-mouse secondary antibody, L-[2,3,4,5-^{3}H]proline, [γ-^{32}P]ATP, and the enhanced chemiluminescence kit were obtained from Amersham Corp. (Amersham, UK). *N,N′*-methylenebisacrylamide, acrylamide, SDS, ammonium persulfate, Temed, and Tween 20 were purchased from Bio-Rad Laboratories (Hercules, CA). Bovine serum albumin, DMSO, β-aminopropionitrile, *N*-ethylmaleimide, and all other chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO).

Culture Conditions

NRK-49F cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in culture flasks and maintained in DMEM (5.5 mM D-glucose) supplemented with 100 i.u./ml penicillin, 100

μg/ml streptomycin and 5% FCS in a humidified 5% CO_2 incubator at 37°C. In some experiments, cells were exposed to serum-free (0.1% FCS) DMEM supplemented with the specific JAK2 inhibitor, AG-490, for 16 h prior to timed exposure to FCS and AGE. AG-490 was dissolved in dimethylsulfoxide (DMSO).

Preparation of AGEs

Bovine serum albumin (BSA) Fraction V (1 mM) was glycated by incubation with 0.5 M glucose in 50 mM potassium phosphate (pH 7.3)/1 mM EDTA under sterile conditions at 37°C for 8 weeks as our previous study [Huang et al., 1999]. Control, non-glycated BSA consisted of the same initial preparations of albumin incubated at 37°C in the same manner, except that no glucose was added.

Production of Collagen

Production of collagen was determined according to a previous study [Peterkofsky and Diegelmann, 1971]. Briefly, 8.0×10^3 cells were transferred to 24-well microplates (Nunclon, Denmark) and maintained in 5% FCS medium for 1 day. After fasting (0.1% FCS) for 48 h, fresh DMEM (5% FCS) with different concentrations of AGE or BSA were added. Cells were grown for an additional 3, 5, and 7 days, and DMEM medium containing ascorbic acid (50 μg/ml), β-aminopropionitrile (β-APN) (80 μg/ml), and 4 μCi ^{3}H-proline (L-[2,3,4,5-^{3}H]proline) were included during the last 24 h. The medium containing non-crosslinked collagen was removed, centrifuged (500g for 10 min) to remove cellular elements, and the supernatant medium proteins precipitated with 10% trichloroacetic acid (TCA) at 4°C overnight. The samples were centrifuged, the pellet washed twice with ethanol, and then resuspended in 50 mM Tris buffer containing $CaCl_2$ (1 mM) and *N*-ethylmaleimide (NEM, 4 mM). The samples were then digested with 20 units of type VII collagenase for 90 min at 37°C (Sigma Chemical Co., St. Louis, MO) and the solution reprecipitated with TCA. The collagenase-sensitive proteins were digested and therefore not precipitable with TCA. The supernatant, containing collagenase-sensitive proteins, was used for liquid scintillation counting. The pellet (collagenase-insensitive proteins) was resuspended and aliquots counted. In addition, TCA-precipitable total ^{3}H-proline incorporation was measured. Briefly, cells grown in 24-well plates were washed. This was followed by protein precipitation with 10% TCA for an additional 15 min. After aspiring TCA, cells were dehydrated with 95% ethanol for 10 min and redissolved in 0.5 M NaOH with 0.1% Triton X-100 at 37°C for 30 min. The lysate was harvested and used for liquid scintillation counting. Data were plotted as cpm/10^4 cells. Each experimental data point represents the mean of duplicate wells from three independent experiments.

Electrophoretic Analysis of Collagens

At the indicated times, cells were labeled with ^{3}H-proline for 24 h. Then, the media was harvested and supplemented with 15 mM NEM, 20 mM EDTA, and 1 mM phenylmethylsulfonylfluoride. ^{3}H-proline-labeled proteins were precipitated by cold absolute ethanol to a final concentration of 33% followed by incubation overnight at 0–4°C. Pellets were dissolved in a Laemmli sample buffer containing 5% mercaptoethanol and boiled for 2 min and the proteins were determined. Ten micrograms of protein were applied to each lane of a 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gel with a 4% stacking gel. Highly purified collagen I was run in parallel. Gels were soaked in Enhance (NEN Life Science Products, Inc., Boston, MA), dried, and fluorographed with Biomax film plus intensifying screens at −70°C.

Preparation of Nuclear Extracts

Nuclear extracts were prepared as our previous study [Huang et al., 1999]. Briefly, cells were maintained in 5% FCS medium for 24 h and starved (0.1% FCS) for another 48 h. Fresh DMEM (5% FCS) with different concentrations of AGE or BSA was then added. At indicated times, cells were harvested and vortexed vigorously. Then, cell lysates were centrifuged and nuclear pellets were resuspended in nuclear extraction buffer. Nuclear proteins were measured by using a Bio-Rad protein assay kit. The extracts were stored at −70°C for further use.

Immunoblotting and Immunoprecipitation

For RAGE protein analysis, 1.5×10^7 serum-deprived cells were treated with BSA or AGE as described above. Total cell lysates were harvested, resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to 0.45 μm Protran membranes (Schieicher and Schuell, Keene, NH). The

membranes were blocked in blocking solution and subsequently probed with anti-RAGE antibody (1 μg/ml). The membrane was incubated in 4000 × diluted HRP-conjugated goat anti-rabbit or anti-mouse secondary antibody. The protein bands were detected using the enhanced chemiluminescence (ECL) system, and the percentage of phosphorylated form of protein was determined using a scanning densitometer.

For JAK/STAT activation assays, 2×10^7 serum-deprived cells were treated with BSA or AGE for indicated times and then harvested. Cell extracts were immunoprecipitated with JAK/STAT monoclonal antibodies and protein A/G-agarose beads. Then, the samples were resolved by SDS-PAGE, and transferred to Protran membranes. The membranes were probed with anti-PY20 (0.75 μg/ml), anti-JAK2 (1:1000), anti-STAT1 (1:1000) and anti-STAT3 (1:1000) antibodies. Immunoreactive proteins were detected with the ECL system as described above.

Synthesis of Oligodeoxynucleotides (ODNs)

The role of RAGE was demonstrated by an antisense ODN approach. For example, a previous study found that antisense RAGE ODN partly prevents AGE-induced NF-κB activation and tissue factor production [Bierhaus et al., 1997]. In contrast, the role of STATs was demonstrated by the decoy ODNs similar to our previous study [Huang et al., 1999]. Double-stranded decoy ODN is a ODN of the same sequence as the cis sequence in the promoter region, thereby inhibiting the binding of the transcription factor to the target gene [Tomita et al., 1997]. Therefore, transcription of the target gene is inhibited. In contrast, mismatched ODN is a control ODN with several mutations in the consensus sequence [Tomita et al., 1997]. The decoy ODN approach has been used in many conditions, including glomerulonephritis [Tomita et al., 1997].

The sequences of phosphorothioate double-stranded ODNs used in this study were synthesized using a DNA/RNA synthesizer (Applied Biosystems Division, Perkin-Elmer Inc., Boston, MA). RAGE antisense ODN (5′-AGCTACTGTCCCCGTTGG-3′) and sense ODN (5′-CCAACGGGGACAGTAGCT-3′) correspond to the region of bp +4 to bp +21 (rat) derived from the cDNA sequences for RAGE (GenBank accession No. L33413). STAT1 and STAT3 ODNs correspond to the high affinity Ly-6E IFN-γ-activated site [Khan et al., 1993] and the acute phase response element in the rat α_2-macroglobulin gene [Campbell et al., 1995], respectively. STAT1 decoy ODN (consensus sequences as the *cis*-element in the promoter region are underlined) comprises 5′-AT<u>ATTCCTGTAAG</u>TG-3′ and 3′-TA<u>TAAGGACATTC</u>AC-5′ while mismatched ODN (MIS1, having three mutations in the consensus sequence) comprises 5′-AT<u>ATTGGAGTAAG</u>TG-3′ and 3′-TA<u>TAACCTCATTC</u>AC-5′. STAT3 decoy ODN comprises 5′-GATCC<u>TTCTGGGAATT</u>CCTAGATC-3′ and 3′-CTAGG <u>AAGACCCTTAA</u>GGATCTAG-5′ while mismatched ODN (MIS3, having three mutations in the consensus sequence) comprises 5′-GATCC<u>TTCTGGGCCGT</u>CCTAGATC-3′ and 3′-CTAGG <u>AAGACCCGGCA</u>GGATCTAG-5′. The decoy ODNs (single-stranded) were annealed for 2 h while the temperature descended from 80 to 25°C.

Transfection of Decoy ODNs Into Cells

Antisense or decoy ODNs dissolved in DMEM were mixed with Lipofectin at a ratio of 1 nmol/μg, The ODN-liposome complexes were added to flasks or 24-well plates. We had determined the specific conditions under which cationic liposomes could be used to successfully transfect the antisense or decoy ODN into NRK-49F cells without causing cellular toxicity.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed as our previous study [Huang et al., 1999]. Briefly, ^{32}P-labeling of STAT1 and STAT3 decoy ODNs were carried out using T4-polynucleotide kinase and [γ-^{32}P] ATP (3,000 Ci/mmol). Labeled DNA was separated from the unincorporated radioactivity. Binding reactions were carried out by adding 5 μg of nuclear protein to 20 μl of binding buffer and [γ-^{32}P]ATP labeled STAT ODN probes. Where indicated, cold competitive oligonucleotides or antibodies were included during the preincubation periods. Samples were incubated at room temperature for 25 min and fractionated by electrophoresis. Following electrophoresis, gels were transferred to 3 MM paper, dried, and exposed to X-ray Hyperfilm-MP at −70°C using an intensifying screen. The results were quantified by a scanning densitometer.

Statistics

The results were expressed as the mean ± standard errors of the mean (SEM). Unpaired

Student's t-tests were used for the comparison between two groups. One-way analysis of variance followed by unpaired t-test was used for the comparison among more than three groups. A P-value less than 0.05 was considered statistically significant.

RESULTS

Time Course and Dose–Response Effects of AGE on Collagen Production

Collagen production was measured by ^{3}H-proline incorporation into cells. We found that AGE (100 μg/ml) increased medium-released (but not cell-associated) collagen at Day 7 (Fig. 1). As shown in Figure 2, AGE also dose-dependently (10–200 μg/ml) enhanced medium-released (but not cell-associated) collagen at Day 7. Thus, an incubation period of 7 days and a dose of 100 μg/ml AGE were selected for further studies.

Antisense RAGE ODNs and Captopril Downregulate RAGE Expression

Because angiotensin II activates NF-κB [Klahr and Morrissey, 2000], while the promoter of RAGE gene contains NF-κB binding sites [Li et al., 1997], we speculated that captopril (an angiotensin II inhibitor) might be effective in reversing AGE-induced RAGE. Thus, as shown in Figure 3A, exposure of NRK-49F cells to 100 μg/ml AGE (but not BSA) for 7 days increased RAGE protein levels. As shown in Figure 3B, the RAGE antisense ODN (0.1–10 μM) and captopril (0.1–10 mM) dose-dependently decreased AGE-induced RAGE protein expression. In contrast, sense ODNs had only minimal effects.

AG-490, Captopril, and Antisense RAGE ODN Inhibit AGE-Induced Tyrosine Phosphorylation of JAK2, STAT1, and STAT3

One of our previous studies has shown that AGE induces the JAK2-STAT1/STAT3 pathway in NRK-49F cells [Huang et al., 1999]. However, engagement of a receptor for AGE (such as RAGE) remains to be determined. Moreover, STAT1 and STAT3 are downstream effectors of JAK2. Thus, to prove the role of RAGE and JAK2 in AGE-induced STAT activation, antisense RAGE ODN and AG-490 were used, where AG-490 is a specific JAK2 inhibitor [Meydan et al., 1996; Kumano et al., 2000; Huang et al., 1999]. Additionally, angiotensin II induces many signal transducers including JAK/STAT [Ruiz-Ortega et al., 2000]. There-

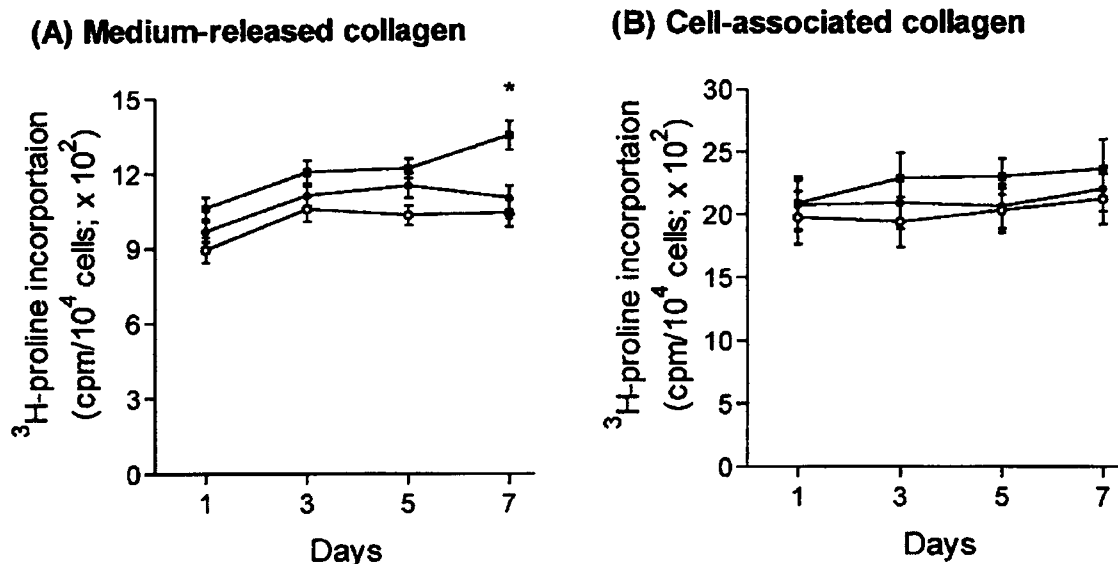

Fig. 1. Time course of AGE effects on collagen production in NRK-49F cells. Serum-deprived NRK-49F cells were treated with 5% FCS (○), AGE (100 μg/ml) (■) and BSA (100 μg/ml) (●) for 1, 3, 5, and 7 days. Collagen production was determined by the incorporation of ^{3}H-proline in NRK-49F cells. AGE significantly increased medium-released collagen (**A**), but not cell-associated collagen (**B**), at Day 7. Results were expressed as the mean ± SEM of three independent experiments. $^*P < 0.01$ versus BSA.

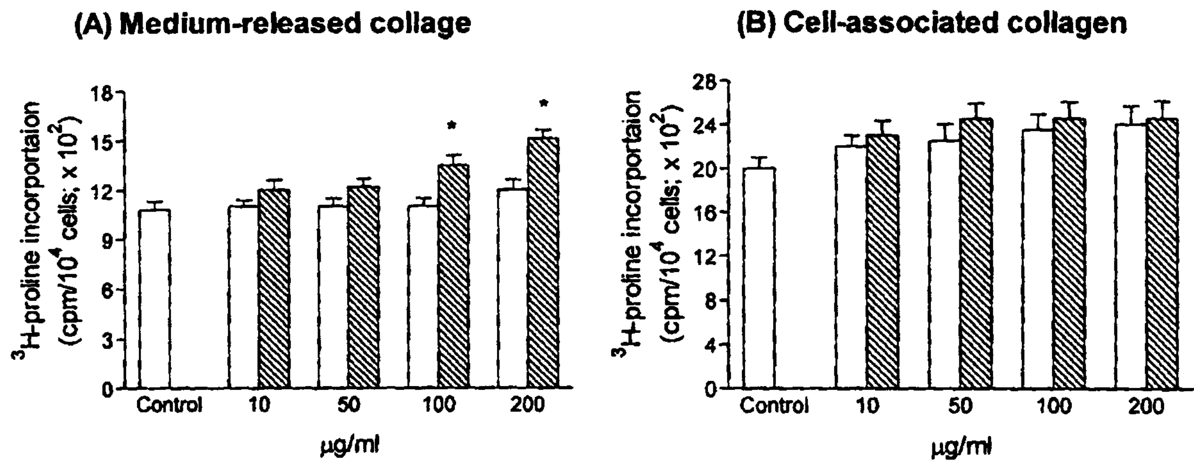

Fig. 2. Dose-dependent effects of AGE on collagen production in NRK-49F cells. Serum-deprived NRK-49F cells were treated with 5% FCS (control), AGE (hatched bars), and BSA (open bars) for 7 days. Collagen production was determined by the incorporation of ^{3}H-proline in NRK-49F cells. AGE dose-dependently increased medium-released collagen (**A**), but not cell-associated collagen (**B**) at Day 7. Results were expressed as the mean $\pm$ SEM of three independent experiments. $^*P < 0.01$ versus BSA.

fore, we speculate that captopril might be effective in reversing AGE-induced JAK/STAT activation. As shown in Figure 4, after incubation with AGE (100 μg/ml) for 0.5 h, tyrosine phosphorylation of JAK2 was increased when compared to control cells incubated with BSA and FCS alone. AG-490 (5 μM) pretretment for 16 h inhibited AGE-enhanced tyrosine phosphorylation of JAK2. Addition of captopril (1 mM) and antisense RAGE ODN (1 μM) also showed similar results. In contrast, sense ODNs had only minimal effects. Tyrosine phosphorylation of STAT1 and STAT3 also showed similar results when cells were treated with AGE for 2 h (Fig. 4).

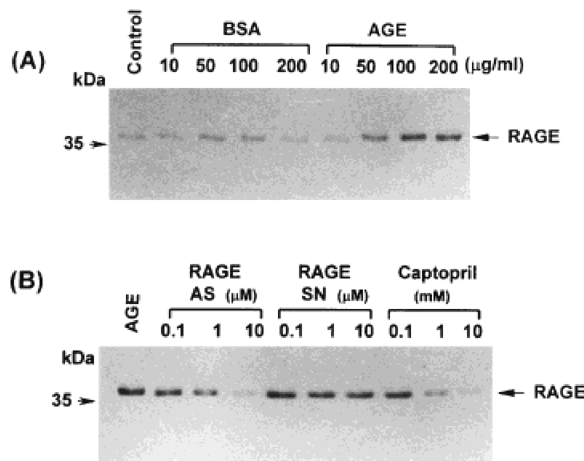

Fig. 3. Effects of antisense RAGE ODN and captopril on AGE-induced RAGE protein expression. (**A**) NRK-49F cells were treated with 10, 50, 100, and 200 μg/ml of AGE or BSA for 7 days. Total cell lysates were separated by 12% SDS-PAGE and analyzed by immunoblotting. (**B**) Cells were treated with antisense RAGE ODN (RAGE AS), sense RAGE ODN (RAGE SN), and captopril in the presence of AGE (100 μg/ml) for 7 days. These are representative experiments, each performed at least three times.

AG-490, Captopril, Antisense RAGE ODN, STAT1 and STAT3 Decoy ODNs Suppress AGE-Induced DNA Binding Activities of STAT1 and STAT3

To test whether the above results for the tyrosine phosphorylation of STAT1 and STAT3 were also observed in protein–DNA binding activities, EMSA was performed. The binding protein complexes were characterized by incubation with a 100-fold molar excess of unlabeled consensus ODNs (Fig. 5A, lane 1). Consistent with the results from the tyrosine phosphorylation assays, the STAT1 and STAT3 binding activities showed a substantial increase upon treatment of the cells with AGE (100 μg/ml) for 2 h (Fig. 5A, lane 4). As shown in Figure 5B, AG-490 (5 μM), captopril (1 mM), and antisense RAGE ODN (1 μM) inhibit AGE-induced STAT1 and STAT3 protein–DNA binding activities. To provide evidence that the decoy ODN prevented the binding of transcriptional factor to its target sites, we also performed EMSAs in the presence of STAT1

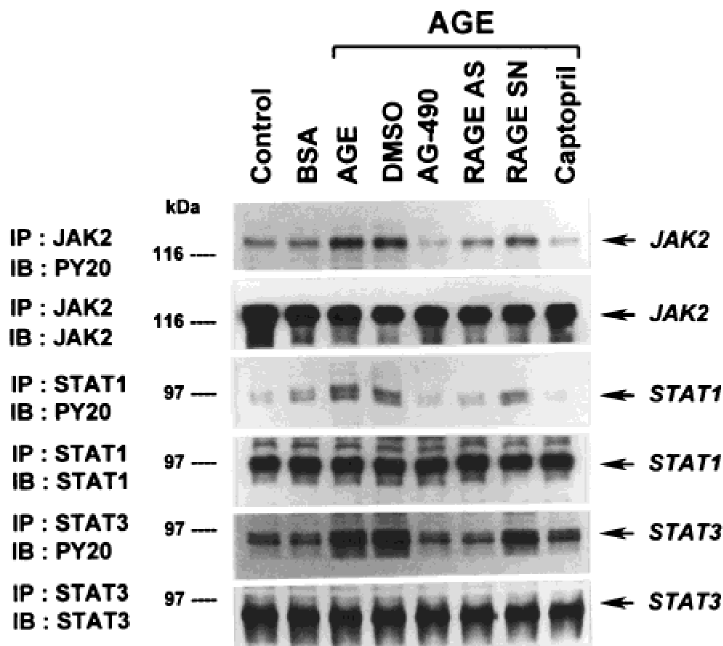

Fig. 4. Effects of AG-490, antisense RAGE ODN, and captopril on AGE-induced tyrosine phosphorylation of JAK2, STAT1, and STAT3. Total cell lysates from NRK-49F cells treated with AG-490 (the specific JAK2 inhibitor, 5 μM), antisense RAGE ODN (RAGE AS, 1 μM), sense RAGE ODN (RAGE SN, 1 μM), and captopril (1 mM) in the presence of 100 μg/ml AGE were immunopreciptated (IP) with anti-JAK2, anti-STAT1, and anti-STAT3 antibodies. Immune complexes were separated by 10% SDS-PAGE and immunoblotted (IB) with either antiphosphotyrosine (PY20) antibodies (upper panels) or antibodies corresponding to the above antibodies (lower panels). DMSO was the solvent used to dissolve AG-490. These are representative experiments, each performed at least for three times.

and STAT3 decoy ODNs and the mismatched control ODNs (Fig. 5B, lane 7 and 8). It was found that AGE-induced STAT1 and STAT3 DNA binding activities were abolished by treatment with 2 μM STAT1 and STAT3 decoy ODNs, respectively. In contrast, sense and mismatched ODNs had only minimal effects.

Effects of AG-490, Captopril, Antisense RAGE ODN, STAT1 and STAT3 Decoy ODNs on AGE-Induced Increase of Collagen Production in NRK-49F Cells

Because the above results demonstrated the effectiveness of JAK2 inhibition (by AG-490) and angiotensin II inhibition (by captopril) on AGE-induced STAT1 and STAT3 activities, AG-490 and captopril were further tested for their effects on AGE-induced collagen production. Moreover, the role of STAT1 and STAT3 in AGE-induced collagen production was tested by the STAT decoys. Figure 6 illustrates the electrophoretic analysis of the ^{3}H-proline-labeled α1 and α2 chains of type I collagen in the conditioned medium of NRK-49F cells cultured in 100 μg/ml BSA or AGE for 7 days. Effects of various inhibitors of STAT1 and STAT3 on type I collagen chains were compared with a highly purified standard (data not shown) and by disappearance of the bands on treatment with collagenase (Fig. 6, lane 1). Thus, AGE increased the production of medium-released type I collagen, which is the major collagen species produced by renal fibroblasts in culture [Müller and Rodemann, 1991]. Meanwhile, captopril significantly reduced AGE-induced production of type I collagen. Similarly, AG-490, antisense RAGE ODN, and STAT1/STAT3 decoy ODNs also inhibited AGE-induced type I collagen production. Additionally, similar results were found in the levels of ^{3}H-proline incorporation into medium-released (but not cell-associated) collagen (Table I).

DISCUSSION

The role of fibroblast-produced collagen in tissue fibrosis has been known for several years [Varga and Jimenez, 1995]. However, the role of renal interstitial fibroblast, which mainly produces type I collagen [Müller and Rodemann, 1991], in renal fibrosis and DN has not been recognized until recently [Eddy, 1996]. One of our previous studies has proven that AGE induces proliferation in NRK-49F cells [Huang et al., 1999]. We have further shown that AGE dose-dependently increased type I collagen production in these cells in the present

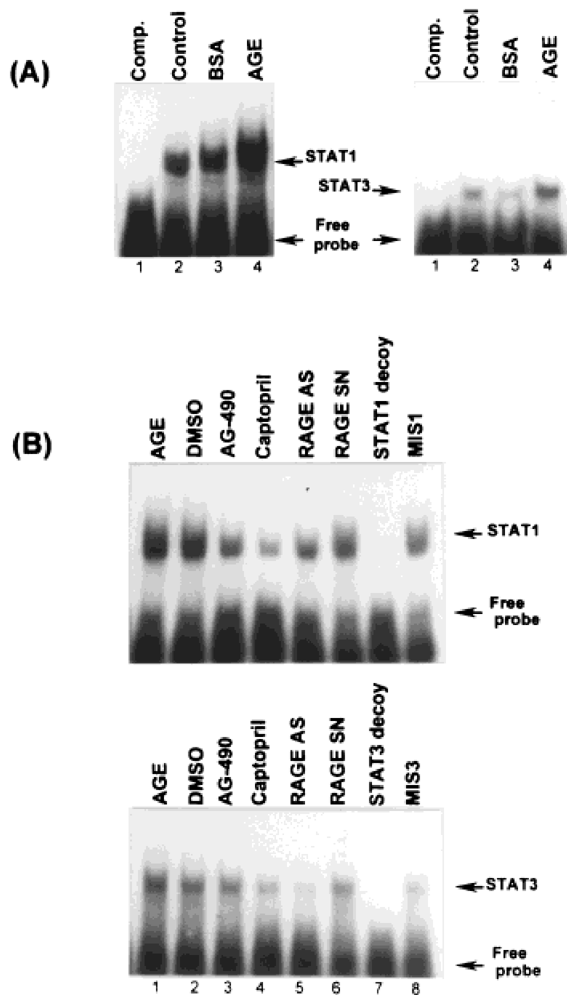

Fig. 5. Electrophoretic mobility shift assay for STAT1 and STAT3 protein-DNA binding activities in NRK-49F cells. (**A**) Serum-deprived cells were treated with 100 μg/ml BSA or AGE for 2 h. Nuclear extracts were prepared and assayed for STAT1 and STAT3 activities, as described under Materials and Methods. (**B**) Nuclear extracts from AGE-treated NRK-49F cells were used as controls (lane 1). Cells were pretreated with DMSO or AG-490 (5 μM) for 16 h prior to exposure to AGE (lane 2 and 3). AGE-stimulated NRK-49F cells were treated with captopril (1 mM) (lane 4) or transfected with antisense RAGE ODN (RAGE AS, 1 μM) (lane 5) and STAT1 or STAT3 decoy ODN (2 μM) (lane 7). In addition, cells transfected with sense RAGE ODN (RAGE SN) and mismatched ODN1 (MIS1) or mismatched ODN3 (MIS3) were analyzed. This is a representative experiment independently performed three times.

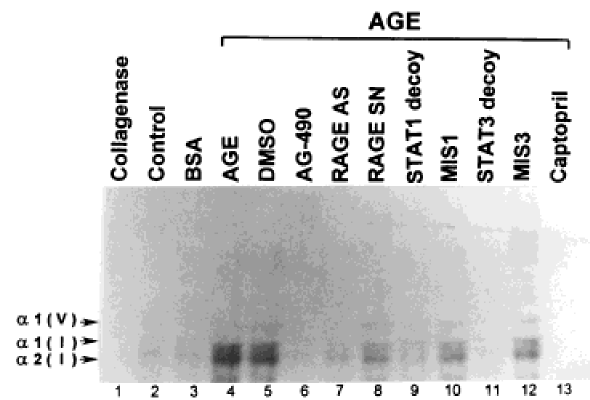

Fig. 6. Electrophoretic analysis of collagen type I production in NRK-49F cells. Cells were treated with the indicated inhibitors in the presence of AGE for 7 days. ^{3}H-proline-labeled proteins were precipitated from the media. Collagenous proteins were separated by 7.5% SDS-PAGE and gel was fluorographed as described in Materials and Methods. α1(I) and α2(I), collagen type I; α1(V), collagen type V. This is a representative of three independently performed experiments.

study. Hence, cellular hyperplasia and overproduction of ECM in renal fibroblasts are associated in this model.

We showed that the AGE-induced effects in NRK-49F cells might be partially mediated by RAGE. Thus, in this study, antisense RAGE ODN reversed AGE-induced production of type I collagen. Moreover, antisense RAGE ODN also reversed AGE-induced tyrosine phosphorylation of JAK2-STAT1/STAT3 and the DNA-protein binding activity of STAT1/STAT3. Thus, it is apparent that the JAK/STAT signaling pathway may be one of the downstream mediators of RAGE in NRK-49F cells.

It is interesting to note that AGE induced RAGE protein synthesis in NRK-49F cells in this study, which may partly explain why AGE-induced adverse effects can propagate for a long time. Furthermore, we showed that antisense RAGE ODN and captopril (an angiotensin II inhibitor) downregulated AGE-induced RAGE overexpression. Thus, we speculate that AGE-induced overexpression of RAGE might be dependent on angiotensin II and the JAK/STAT pathway, based on the following reasons. First, we have shown that STAT is a downstream mediator of the AGE/RAGE interaction. Second, STAT are also one of the downstream mediators of angiotenin II receptor type I [Ruiz-Ortega et al., 2000]. Third, there are STAT-binding sites in the RAGE gene promoter (Matinspector program) [Quandt et al., 1995]. Thus, studies are underway to test these speculations.

We also found that the JAK2-STAT1/STAT3 pathway was necessary for AGE-induced type I

TABLE I. Effects of AG-490, Captopril, Antisense RAGE ODN, STAT1 and STAT3 Decoy ODNs on AGE-Induced Collagen Synthesis in NRK-49F Cells

Treatment	MR collagen (% of control)	CA collagen (% of control)
AGE (100 μg/ml) [control]	100	100
+DMSO	98 ± 6.8	95 ± 4.5
+AG-490 (5 μM)	72 ± 6.4*	93 ± 6.7
+Captopril (1 mM)	75 ± 5.5*	91 ± 8.1
+Antisense RAGE ODN (1 μM)	78 ± 4.7*	89 ± 5.3
+Sense RAGE ODN (1 μM)	92 ± 8.2	98 ± 5.6
+STAT1 Decoy ODN (2 μM)	71 ± 4.2*	91 ± 8.1
+Mismatched ODN1 (2 μM)	89 ± 8.3	95 ± 6.8
+STAT3 Decoy ODN (2 μM)	72 ± 5.0*	90 ± 3.6
+Mismatched ODN3 (2 μM)	94 ± 6.2	90 ± 6.6
Medium alone	76 ± 6.7*	96 ± 5.1
BSA (100 μg/ml)	75 ± 6.6*	96 ± 6.4

NRK-49F cells were exposed to AGE (100 μg/ml) or BSA (100 μg/ml) for 7 days. The incorporation of ^{3}H-proline into medium-released (MR) collagen and cell-associated (CA) collagen (cpm/10^4 cells) were determined as described in Materials and Methods. Values are expressed relative to the control value, which is set at 100. Each value is the mean ± SEM for four cultures pooled from three experiments. *Significant effect of the agonist compared with control, $P < 0.05$.

collagen production in NRK-49F cells. This finding was achieved using a specific JAK2 inhibitor (AG-490) and a versatile decoy ODN approach, which was similar to the one used in our previous study. Thus, AG-490 and STAT1/STAT3 decoy ODN reversed AGE-induced STAT1 and STAT3 tyrosine phosphorylation and DNA binding activities while inhibiting the production of type I collagen in NRK-49F cells.

AGE might induce collagen production either directly or indirectly by inducing other cytokines. There are three reasons to believe there is a direct pathway. First, STAT is responsible for AGE-induced proliferation in NRK-49F cells [Huang et al., 1999], while collagen production is increased in the context of increased cellular numbers in fibroblasts [Makela et al., 1990]. Second, there are no STAT-binding sites in the type I collagen gene promoter (Matinspector program) [Quandt et al., 1995]. However, STAT may induce type I collagen production by inhibiting AP-1 [Horvai et al., 1997], because there are inhibitory AP-1 binding sites in the type I collagen gene promoter in fibroblasts [Lee et al., 1998]. However, the exact mechanism of transcriptional activation of type I collagen remains to be studied.

Because AGE activates NF-κB [Schmidt et al., 1996], while NF-κB inhibits the expression of type I collagen gene [Rippe et al., 1999], it is surprising that AGE also induces collagen production. However, this paradox can be explained by the following observations. First, NF-κB is frequently co-expressed with angiotensin II and collagen in renal fibrosis, which is characterized by increased collagen [Klahr and Morrissey, 2000]. Indeed, both angiotensin II and NF-κB have been proposed to play a role in renal fibrosis [Klahr and Morrisse, 2000]. Second, NF-κB induces fibroblast proliferation and activates angioteninogen, which upregulates angiotensin II [Klahr and Morrissey, 2000]. Because collagen production is increased in the context of increased cellular numbers in fibroblasts [Makela et al., 1990] and angiotensin II induces proliferation and collagen production in fibroblasts [Ruiz-Ortega et al., 2000], AGE-induced NF-κB might induce collagen by increasing cell numbers and angiotensin II in these cells. Third, AGE-induced STAT may inhibit NF-κB [Wang et al., 2000], thereby reversing NF-κB-inhibitable collagen production. Of course, the above possibilities require further confirmations.

Evidence for the indirect pathway is that STAT3 may activate angiotensinogen gene [Mascareno et al., 1998], while fibroblasts have angiotensin converting enzyme, angiotensin receptor and the ability to produce angiotensin in vitro [Hafizi et al., 1998; Sun et al., 1997; Dostal et al., 1992]. Based on our present finding that captopril reversed AGE-induced collagen, it is reasonable to speculate that angiotenin II may mediate STAT-induced

collagen production. Indeed, angiotensin II has been implicated in renal fibrosis [Klahr and Morrissey, 2000] and shown to induce proliferation and collagen production in renal interstitial fibroblasts [Ruiz-Ortega et al., 2000]. Additionally, AGE induces platelet-derived growth factor [Throckmorton et al., 1995], while platelet-derived growth factor induces type I collagen production [Ivarsson et al., 1998]. Another relevant cytokine is TGF-β, which is the most important inducer of extracellular matrix in renal fibrosis [Border and Noble, 1994]. However, AGE did not induce TGF-β in NRK-49F cells in this study (data not shown). This observation is similar to our previous study, which showed that high glucose did not induce TGF-β in the distal tubule-like MDCK cells [Yang et al., 1998].

The therapeutic effect of ACEI in DN is mainly due to the inhibition of angiotensin II [Kshirsagar et al., 2000]. Additionally, it has been attributed to both hemodynamic and non-hemodynamic mechanisms [Wolf and Ziyadeh, 1997; Ruiz-Ortega et al., 2000]. For example, one of our previous studies have shown that captopril reversed high glucose-induced effects in the proximal tubule-like LLC-PK_1 cells [Guh et al., 1996]. In the present study, we also showed that captopril reversed AGE-induced JAK2-STAT1/STAT3 activation in NRK-49F cells. This observation has been corroborated by a recent study, which found that ACEI reversed angiotensin II-induced proliferation in fibroblasts partly by inhibiting the JAK/STAT pathway [van Eickels et al., 1999]. Thus, angiotensin II may participate in AGE-induced JAK/STAT activation in these cells. Interestingly, angiotensin II auto-induces angiotensinogen by activating STAT3 and NF-κB, thereby creating a positive feedback loop [Mascareno et al., 1998; Klahr and Morrissey, 2000]. Thus, studies are underway to determine whether AGE affects angiotensinogen expression and whether AGE-induced JAK/STAT signaling is dependent on angiotensin II.

In conclusion, AGE induces RAGE and then activates downstream JAK/STAT signal transducers. Additionally, RAGE and the JAK2-STAT1/STAT3 pathway were involved in AGE-induced collagen production in NRK-49F cells. Moreover, captopril was able to reverse AGE-induced collagen production by attenuating RAGE expression and JAK2-STAT1/STAT3 activities.

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