Sp1-like activity mediates angiotensin-II-induced plasminogen-activator inhibitor type-1 (*PAI-1*) gene expression in mesangial cells

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Angiotensin II (Ang II) up-regulates plasminogen-activator inhibitor type-1 (*PAI-1*) expression in mesangial cells to enhance extracellular matrix formation. The proximal promoter region (bp –87 to –45) of the human *PAI-1* gene contains several potent binding sites for transcription factors [two phorbol-ester-response-element (TRE)-like sequences; D-box (–82 to –76) and P-box (–61 to 54), and one Sp1 binding site-like sequence, Sp1-box 1 (–72 to –67)]. We studied this region to determine the transcription factor(s) that mediates Ang-II-induced transcriptional activation of the *PAI-1* gene. Various double-stranded decoy oligodeoxynucleotides (ODNs) corresponding to various sequences in the proximal promoter region were transfected to mesangial cells to examine the effects on Ang-II-induced *PAI-1* mRNA expression. Transfection with the full-length decoy (bp –87 to –45, D-P-ODN) markedly attenuated Ang-II-induced *PAI-1* mRNA expression by up to 70%. Transfection with D-ODN (–87 to –71) and P-ODN (–66 to –45), which correspond to each of the two TRE-like sequences, did not attenuate the expression. Gel-shift assays using nuclear extracts prepared

from Ang-II-treated mesangial cells and D-P-ODN showed three specific complexes. The major complex was supershifted by anti-Sp1 antibody. The methylation-interference experiment demonstrated that human recombinant Sp1 bound to the so-called GT box (TGGGTGGGGCT, –78 to –69), which contains the Sp1-box 1. The complex that migrated with anti-Sp1 antibody was enhanced in the cells treated with Ang II. Further, D-Sp1-ODN (–85 to –63) containing the GT box attenuated up-regulation of *PAI-1* mRNA expression induced by Ang II to a level ($68 \pm 9\%$ inhibition) comparable to D-P-ODN, whereas ODN with four mutations in the GT box had no effect. Our findings suggest that binding of Sp1 or an Sp1-like transcription factor to the GT box in the *PAI-1* promoter up-regulates *PAI-1* gene transcription in mesangial cells stimulated with Ang II. This transcription-factor binding site may be targeted to control Ang-II-dependent extracellular matrix formation by mesangial cells.

Key words: double-stranded decoy oligodeoxynucleotide, extracellular matrix, transcription, plasmin system.

INTRODUCTION

Mesangial expansion due to an accumulation of extracellular matrix is a hallmark of progressive glomerular diseases. Numerous studies indicate that angiotensin II (Ang II) mediates extracellular-matrix accumulation by a mechanism independent of its vasopressor effect [1]. Ang II stimulates production of extracellular-matrix proteins in glomerular mesangial cells, which are the major cells to produce mesangial extracellular matrix. Ang II has also been shown to reduce the degradation of matrix proteins by up-regulating plasminogen-activator inhibitors [2,3]. Recent studies demonstrate that plasmin is a major regulator of mesangial cell matrix turnover [4]. Plasmin generation is regulated by a balance between plasminogen activator and plasminogen-activator inhibitors. Glomeruli isolated from animal models of proliferative glomerulonephritis showed an increase in plasminogen-activator inhibitor type-1 (*PAI-1*) deposition in the extracellular matrix, which suggests that *PAI-1* is the one of key factors associated with mesangial matrix accumulation [5]. The expression of the *PAI-1* mRNA in mesangial cells has been shown to be up-regulated by Ang II, which activates the transcription of the *PAI-1* gene [6]. We have demonstrated previously that the protein kinase C (PKC)-dependent pathway is involved in the up-regulation of *PAI-1* mRNA [7]. Further,

PKC-activating phorbol ester markedly up-regulates *PAI-1* mRNA expression in mesangial cells.

Phorbol esters have been reported to induce *PAI-1* gene transcription to up-regulate the mRNA level in many cells. Phorbol esters regulate the expression of many genes through the phorbol ester-response elements (TREs). The proximal promoter region of the human *PAI-1* gene close to the TATA box contains two TRE-like elements located at bp –82 to –76 (designated D-box) and –61 to –54 (P-box) upstream of the cap site [8]. The c-Jun homodimer (activator protein 1, AP-1) and c-Jun/c-Fos heterodimer bind to the two TRE-like sequences to mediate phorbol ester response. There are two Sp1 binding site-like sequences at bp –72 to –67 (Sp1-box 1) and –45 to –40 (Sp1-box 2) in the proximal promoter region. Sp1 is a general transcription factor that binds to the so-called GC box and is expressed ubiquitously in many tissues [9]. The functional significance of these TRE-like and Sp1-box elements in Ang-II-induced *PAI-1* mRNA up-regulation has not been identified. This study examined the molecular mechanisms by which Ang II induces up-regulation of *PAI-1* gene expression. For this purpose, a series of decoy oligodeoxynucleotides (ODNs) targeting the promoter region, TRE-like boxes and the Sp1 box of the human *PAI-1* gene was constructed and used to manipulate Ang-II-mediated transcriptional changes. Specific binding of the tran-

Abbreviations used: Ang II, angiotensin II; *PAI-1*, plasminogen-activator inhibitor type 1; PKC, protein kinase C; TRE, phorbol ester-response element; ODN, oligodeoxynucleotide; DIG, digoxigenin; DTT, dithiothreitol; AP, activator protein; MAPK, mitogen-activated protein kinase.

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scriptional factors to these ODNs was further evaluated by gel-shift and supershift assays.

EXPERIMENTAL

Mesangial cell culture

Rat mesangial cells were prepared as described previously [7]. Briefly, kidney cortices were isolated from 7-week-old male Sprague–Dawley rats. They were minced and passed through a series of sieves to isolate the glomeruli. Isolated glomeruli were treated with 1 mg/ml collagenase (Wako, Osaka, Japan) for 30 min, resuspended in RPMI 1640 medium containing 17% fetal bovine serum (Gibco-BRL, Grand Island, NY, U.S.A.), 0.1 units/ml insulin (Sigma, St. Louis, MO, U.S.A.) and antibiotics, and seeded in 100-mm plastic tissue-culture dishes. Outgrowing mesangial cells were maintained in the same medium, characterized by immunohistochemical staining of desmin, α -smooth-muscle actin and cytokeratin, and by low-density lipoprotein uptake. The cells were used in experiments between passages 4 and 7. The expression of Ang-II receptor was confirmed at each passage by a ^{125}I -Ang-II-binding assay.

For Ang-II stimulation, cells were rendered quiescent by culturing in serum-free medium for 24 h, and then stimulated with 100 nM Ang II (Sigma) for 3 or 6 h. In experiments using decoy ODNs, the cells were pretreated with ODN–lipofectin complex (molar ratio, 1:1; Gibco-BRL) at the indicated concentrations in a serum-free medium for 24 h as described elsewhere [10]. They were then stimulated with 100 nM Ang II for 6 h.

Northern blotting

An 877-bp fragment of rat PAI-1 cDNA and a 476-bp fragment of β -actin cDNA were labelled with digoxigenin (DIG) and used as probes for Northern blotting as described previously [7].

Total RNA was isolated using Isogen (Wako), 1 ml of which was added to mesangial cells cultured in a 60-mm dish. After cell lysis, the sample was collected in a tube, and total RNAs were extracted by chloroform and centrifuged. RNAs were precipitated with isopropanol, rinsed in 75% ethanol and resuspended in an appropriate amount of diethyl pyrocarbonate treated water.

Total RNA (10–20 μg) was fractionated by electrophoresis on a 1% agarose gel. The RNA was then transferred from the gel

to a Hybond N⁺ nylon membrane (Amersham, Little Chalfont, Bucks., U.K.) and immobilized by UV cross-linking. The membrane was prehybridized in a solution containing 4 $\times$ SSCP (where 1 $\times$ SSCP is 0.12 M NaCl/0.015 M sodium citrate/0.015 M Na₂HPO₄/0.005 M NaH₂PO₄), 1 $\times$ Denhardt's solution, 1% SDS, 50% formamide and 100 $\mu\text{g}/\text{ml}$ salmon sperm DNA at 42 °C for 3 h. Hybridization was then performed in a solution containing 4 $\times$ SSCP, 1 $\times$ Denhardt's solution, 1% SDS, 5% dextran sulphate, 50% formamide, 100 $\mu\text{g}/\text{ml}$ salmon sperm DNA and denatured DIG-labelled probe at 42 °C for 16 h. After hybridization, the membrane was washed twice with 1 $\times$ SSC (0.15 M NaCl/0.015 M sodium citrate) containing 0.1% SDS at room temperature for 15 min and then washed twice with 0.2 $\times$ SSC containing 0.1% SDS at 42 °C for 15 min. The hybridized probe was detected using a DIG-detection kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

Preparation of nuclear extract

After stimulating rat mesangial cells with 100 nM Ang II for 3 h, the cells were trypsinized and washed with PBS. The cells were suspended in a buffer [10 mM Hepes (pH 7.6) 15 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF and 10 $\mu\text{g}/\text{ml}$ leupeptin] and collected by brief centrifugation at 2000 *g*. The cells were lysed in the same buffer containing 0.2% Nonidet P40 and centrifuged again. The nuclei were isolated by suspending the pellet in a sucrose buffer [0.25 M sucrose, 10 mM Hepes (pH 7.6) 15 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 10 $\mu\text{g}/\text{ml}$ leupeptin] and centrifuging at 2000 *g*. The nuclear protein was extracted by suspending the pellet in an extraction buffer [50 mM Hepes (pH 7.6) 400 mM KCl, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 10 $\mu\text{g}/\text{ml}$ leupeptin] and incubating at 4 °C for 30 min. Insoluble materials were removed by centrifugation at 1000 *g* for 15 min.

Gel-shift assay

Gel-shift assay was performed using a DIG gel-shift kit (Boehringer Mannheim). Briefly, double-stranded ODN probes, as described in Figure 1, were 3'-end labelled with DIG using terminal transferase according to the manufacturer's instructions.

Proximal promoter region of human PAI-1 gene

Figure 1 Sequence of proximal promoter of the human PAI-1 gene and ODNs used in the experiments

The proposed AP-1 and Sp1 binding sites are underlined according to the method of Knudsen et al. [8]. The sequences of ODNs used as decoys and for gel-shift assays are shown. Underlined bases indicate mutations created in the respective ODNs.

The labelled probes (20 fmol) were incubated with the nuclear protein preparation in a binding buffer [20 mM Hepes (pH 7.6) 30 mM KCl, 0.2 % (w/v) Tween 20, 1 mM EDTA, 1 mM DTT, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 25 ng/ μl poly d(I-C), 25 ng/ μl poly d(A-T) and 50 ng/ μl poly L-lysine] at room temperature for 30 min. For gel supershift assay, antisera to Sp1, Sp3, AP-1 or AP-2 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) were added to the mixture and kept on ice for 1 h. After incubation, the protein–DNA complexes were separated by 5 % non-denaturing acrylamide gel electrophoresis. The protein–DNA complexes were transferred to a nylon membrane by electroblotting and were detected using the DIG-detection kit.

Methylation interference

A synthesized sense strand of D-P-ODN (the ODN spanning from D-box to P-box, see Figure 1) was 3'-end labelled with DIG by chemical modification (Japan Bioservice, Saitama, Japan) and then made double-stranded with an unlabelled antisense strand. The dimethyl sulphate reaction was started by adding 1 μl of dimethyl sulphate to a buffer containing 50 mM sodium acetate (pH 8.0), 1 mM EDTA and 33 pmol of the ODN, in a volume of 200 μl . After incubation at room temperature for 10 min, the reaction was stopped by adding 50 μl of the stop solution (1.5 M sodium acetate, pH 8.0, and 1 M β -mercaptoethanol). The ODN was precipitated with 750 μl of ethanol. The precipitate was resuspended in TE buffer (50 mM Tris/HCl, pH 8.0, and 1 mM EDTA) containing 100 mM NaCl. The ODN (200 fmol) was incubated with 1 fpu (footprint unit) of recombinant human Sp1 (Promega, Madison, WI, U.S.A.) in the gel-shift binding buffer for 30 min. The Sp1–ODN complex was separated by electrophoresis on a 5 % non-denaturing acrylamide gel and transferred to DEAE paper by electroblotting. The DEAE paper was washed three times with TE buffer containing 50 mM NaCl. The labelled ODN was extracted with TE buffer containing 1 M NaCl and precipitated with ethanol. The precipitate was suspended in 1 M piperidine, incubated at 90 °C for 30 min and lyophilized. The digested ODN was electrophoresed on a 12 % sequencing gel, transferred to a nylon membrane and detected using the DIG-detection kit.

Results are presented as means $\pm$ S.D. Student's *t* test was used for comparisons, with *P* < 0.05 considered statistically significant. Each experiment was performed at least in triplicate.

RESULTS

PAI-1 gene promoter activity was studied by transfecting mesangial cells with double-stranded decoy ODNs to inhibit specific transcription factors. This experimental approach overcame the technical difficulties of transfecting mesangial cells with plasmid vectors to perform a functional reporter gene assay. Figure 1 shows the sequence of the human *PAI-1* proximal promoter region [8] and the sequences of the decoy ODNs used in this study. The region between bp –79 and –44 in the human *PAI-1* gene is identical with the corresponding regions in the mouse and rat genes. The D-box and P-box are similar to the binding sites for AP-1 (TREs). The sequences with similarity to the general transcription factor Sp1 binding site are designated Sp1-box 1 and Sp1-box 2.

Initially, we assessed the effect of the decoy ODN spanning from D-box to P-box (D-P-ODN). D-P-ODN dose-dependently inhibited the Ang-II-induced *PAI-1* mRNA up-regulation in rat mesangial cells (Figure 2). To examine which portion of D-P-ODN is responsible for the inhibition, ODNs corresponding to the D-box (D-ODN), the P-box (P-ODN) and D-P-ODN with

Figure 2 Effect of the decoy D-P-ODN on Ang-II-induced *PAI-1* mRNA expression

Mesangial cells were transfected with D-P-ODN at different concentrations in a serum-free medium for 24 h, and then stimulated with 100 nM Ang II for 6 h. The *PAI-1* mRNA expression was analysed by Northern blotting using rat *PAI-1* probe. Lane 1, control, no treatment; lane 2, Ang II; lane 3, 1 μM D-P-ODN + Ang II; lane 4, 5 μM D-P-ODN + Ang II.

Figure 3 Effects of the decoy M D-P-ODN, D-ODN and P-ODN on Ang-II-induced *PAI-1* mRNA expression

(A) Northern blotting. Mesangial cells were transfected with 5 μM D-P-ODN, M D-P-ODN, D-ODN or P-ODN in a serum-free medium for 24 h, and then stimulated with 100 nM Ang II for 6 h. The *PAI-1* mRNA expression was analysed by Northern blotting using rat *PAI-1* probe. Lane 1, control, no treatment; lane 2, Ang II; lane 3, D-P-ODN + Ang II; lane 4, M D-P-ODN + Ang II; lane 5, D-ODN + Ang II; lane 6, P-ODN + Ang II. (B) Gel-shift assay. 3'-End labelled D-P-ODN was incubated with nuclear extracts prepared from mesangial cells in the absence or presence of a 100-fold excess of unlabelled ODN. The protein–DNA complexes were separated by 5 % non-denaturing acrylamide gel electrophoresis. Lane 1, control, no competitor; lane 2, 100-fold excess of unlabelled D-P-ODN; lane 3, 100-fold excess of unlabelled M D-P-ODN; lane 4, 100-fold excess of unlabelled D-ODN; lane 5, 100-fold excess of unlabelled P-ODN.

mutations at the Sp1-box 1 (M D-P-ODN) were constructed (Figure 1). Cells transfected with the D-ODN and P-ODN showed no inhibition but rather enhanced signals of *PAI-1* mRNA when treated with Ang II (Figure 3A). Transfection with M D-P-ODN inhibited the up-regulation induced by Ang II to a level comparable with that with the D-P-ODN. These data suggest that (i) both D- and P-boxes are necessary for inducing *PAI-1* mRNA up-regulation by Ang II, or (ii) the mutation created in the Sp1-box 1 is not effective for inhibiting Sp1

Figure 4 Effects of the decoy Sp1-ODN on Ang-II-induced PAI-1 mRNA expression

(A) Northern blotting. Mesangial cells were transfected with 5 μ M Sp1-ODN or M Sp1-ODN in a serum-free medium for 24 h, and then stimulated with 100 nM Ang II for 6 h. The PAI-1 mRNA expression was analysed by Northern blotting using rat PAI-1 probe. Lane 1, control, no treatment; lane 2, Ang II; lane 3, Sp1-ODN + Ang II; lane 4, M Sp1-ODN + Ang II. (B) Gel-shift assay. 3'-End labelled D-P-ODN was incubated with nuclear extract prepared from mesangial cells in the absence or presence of a 100-fold excess of unlabelled ODN. The protein-DNA complexes were separated by 5% non-denaturing acrylamide gel electrophoresis. Lane 1, control, no competitor; lane 2, 100-fold excess of unlabelled D-P-ODN; lane 3, 100-fold excess of unlabelled Sp1-ODN.

binding. To address these questions, a gel-shift assay was performed using mesangial-cell nuclear extracts and the D-P-ODN. The gel-shift assay showed at least three specific bands, which were abolished by the addition of an excess of unlabelled D-P-ODN (designated bands 1–3 from the top, Figure 3B). Addition of the unlabelled M D-P-ODN to the reaction mixture abolished complex formation of all the three bands. However, addition of D-ODN or P-ODN exhibited only weak competition for these bands. These data indicate that M D-P-ODN has similar capability to D-P-ODN in competing for binding with transcription factors.

Since the decoy ODN with mutations in the Sp1-box 1 (M D-P-ODN) failed to inhibit transcription-factor binding, we synthesized another decoy, Sp1-ODN, to inhibit Sp1 transcriptional activity. Mesangial cells transfected with Sp1-ODN showed a weak attenuation of Ang-II-induced PAI-1 mRNA up-regulation (about 30% reduction, Figure 4A), whereas the mutated control M Sp1-ODN had little effect on the expression. The gel-shift assay showed that intensities of the three bands were decreased by the addition of excess Sp1-ODN, but it did not completely compete out these bands as D-P-ODN did (Figure 4B). Thus the affinity of Sp1-ODN for these factors was much lower than that of D-P-ODN. These data indicate that the weak affinity of Sp1-ODN for transcription factor resulted in weak inhibition of Ang-II-induced PAI-1 mRNA up-regulation.

To identify the transcription factors that bind to D-P-ODN we performed gel supershift assays with antibodies to AP-1, AP-2

Figure 5 Molecular interaction of Sp1 or Sp1-like activity in mesangial cells with the D-P-ODN

(A) Gel supershift assay. 3'-End labelled D-P-ODN was incubated with nuclear extracts prepared from mesangial cells. Then, an antibody to Sp1, AP-1 or AP-2 was added to the reaction mixtures. The protein-DNA complexes were separated by 5% non-denaturing acrylamide gel electrophoresis. Lane 1, control, no antibody; lane 2, anti-Sp1 antibody; lane 3, anti-AP-1 antibody; lane 4, anti-AP-2 antibody. (B) Competition of gel shift by D-P-ODN or Sp1 consensus ODN. 3'-End labelled D-P-ODN was incubated with nuclear extracts from mesangial cells in the presence of various concentrations of D-P-ODN or Sp1 consensus ODN. The protein-DNA complexes were separated by 5% non-denaturing acrylamide gel electrophoresis. Lane 1, control, no competitor; lane 2, 80 fmol of D-P-ODN (4-fold); lane 3, 400 fmol of D-P-ODN (20-fold); lane 4, 2000 fmol of D-P-ODN (100-fold); lane 5, 80 fmol of Sp1 consensus ODN (4-fold); lane 6, 400 fmol of Sp1 consensus ODN (20-fold); lane 7, 2000 fmol of Sp1 consensus ODN (100-fold).

and Sp1. The antibody to Sp1 supershifted band 1 (Figure 5A). The anti-AP-1 antibody also yielded a supershifted band, although we could not identify which of the three bands had been supershifted. The anti-AP-2 antibody did not cause supershifting. A competition assay using a putative Sp1 consensus sequence abolished binding of the three bands with an affinity comparable with the D-P-ODN (Figure 5B). These results indicate that Sp1 or an Sp1-like factor binds to a region of the *PAI-1* gene corresponding to the D-P-ODN.

To define the binding site of Sp1 in the *PAI-1* gene, we performed a methylation-interference experiment using DIG-labelled D-P-ODN and human recombinant Sp1. The methylated ODN interfered with Sp1 binding from bp -78 to -69 (Figure 6). Thus Sp1 binds to the ODN at the sequence TGGGTGGGCT, which is the so-called GT-box sequence and contains Sp1-box 1 in the *PAI-1* gene. To verify the binding of Sp1 or Sp1-like activity to the GT box, D-Sp1-ODN, which contains the GT box, and a control, M D-Sp1-ODN, with four mutations in the GT box (TGGGTGGGCT → TGAATTCGGCT, with the changes underlined), were synthesized. When D-Sp1-ODN was incubated with mesangial-cell nuclear extracts, three complexes were observed, as shown earlier with D-P-ODN, although the intensities of bands 2 and 3 were weak (Figure 7). These three bands were competed out when a 100-fold excess of D-Sp1-ODN

3'-End labelled D-P-ODN was methylated and incubated with recombinant human Sp1. The protein-DNA complexes were isolated and the DNA was digested by the Maxam-Gilbert reaction. The DNA fragments were separated by electrophoresis on a 12% sequence gel. Lane 1, control, no protein incubation; lane 2, incubated with recombinant human Sp1.

The nature of the band-1 complex in Ang-II-induced PAI-1 mRNA up-regulation was studied by a gel-shift assay. The intensity of the band-1 complex in nuclear extract from rat mesangial cells treated with Ang II for 3 h was compared with that from untreated control cells. The intensity of band-1 complex was substantially increased after 3 h of Ang II treatment (Figure 8). Densitometric analysis revealed a 2.36 ± 0.05 -fold increase in

3'-End labelled D-Sp1-ODN was incubated with nuclear extracts from mesangial cells in the absence or presence of 100-fold excess of unlabelled ODN or anti-Sp1 antibody. The protein-DNA complexes were separated by 5% non-denaturing acrylamide gel electrophoresis. Lane 1, control, no competitor; lane 2, 100-fold excess of unlabelled D-Sp1-ODN; lane 3, 100-fold excess of unlabelled M D-Sp1-ODN; lane 4, gel supershift assay with anti-Sp1 antibody.

3'-End labelled D-Sp1-ODN was incubated with nuclear extracts of Ang-II-treated mesangial cells. The protein-DNA complexes were separated by 5% non-denaturing acrylamide gel electrophoresis. (Although not shown, Western-blot analysis of the nuclear extract from mesangial cells showed no apparent change in the Sp1 protein after the treatment with Ang II.) Lane 1, control, untreated cells; lane 2, Ang-II-treated cells.

Sp1-like-factor binding by Ang II compared with non-treated control, whereas the intensities of bands 2 and 3 were comparable. The Ang-II-responsive component of band 1 was evaluated further. When an antibody to Sp3, which also belongs to the Sp1 family of transcription factors, was added to the system, it supershifted band 1. When both anti-Sp1 and anti-Sp3 antibodies were added to the system, the band-1 complex disappeared completely (Figure 9A). The results indicate that band 1 consists mainly of Sp1 and Sp3. Then the amount of Sp1 and Sp3 in the nuclear extracts from Ang-II-treated and non-treated cells was compared by adding each antibody to the system and the intensity of remaining band 1 was measured. When anti-Sp1 antibody was added to the system there was no significant difference in the intensities of remaining band 1 (Sp3 or Sp3-like)

(A) Gel supershift assay. 3'-End labelled D-Sp1-ODN was incubated with nuclear extracts prepared from mesangial cells. Then, an antibody to Sp1 and/or Sp3 was added to the reaction mixtures. The protein-DNA complexes were separated by 5% non-denaturing acrylamide gel electrophoresis. Lane 1, control, no antibody; lane 2, anti-Sp1 antibody; lane 3, anti-Sp3 antibody; lane 4, anti-Sp1 and anti-Sp3 antibodies. **(B)** Comparison of Sp1(-like activity) and Sp3(-like activity) in nuclear extracts from Ang-II-treated cells and non-treated cells. 3'-End labelled D-Sp1-ODN was incubated with nuclear extracts from mesangial cells. Then, an antibody to Sp1 or Sp3 was added to the reaction mixtures. The protein-DNA complexes were separated by 5% non-denaturing acrylamide gel electrophoresis. Lanes 1 and 2, supershifted with anti-Sp1 antibody; lanes 3 and 4, supershifted with anti-Sp3 antibody. Lanes 1 and 3, control, non-treated cells; lanes 2 and 4, Ang-II-treated cells.

Figure 10 Effect of the decoy D-Sp1-ODN on Ang-II-induced PAI-1 mRNA expression

(A) Basal PAI-1 mRNA expression. (B) Ang-II-induced PAI-1 mRNA expression. Mesangial cells were transfected with D-Sp1-ODN or M D-Sp1-ODN by incubation with indicated concentrations of the ODN in a serum-free medium for 24 h, and then stimulated with vehicle (Control, A) or 100 nM Ang II (B) for 6 h. The PAI-1 mRNA expression was analysed by Northern blotting using rat PAI-1 probe. Lanes 1, control, no ODN treatment; lanes 2, 1 μ M D-Sp1-ODN; lanes 3, 5 μ M D-Sp1-ODN; lanes 4, 1 μ M M D-Sp1-ODN; lanes 5, 5 μ M M D-Sp1-ODN.

between the nuclear extract from Ang-II-treated and non-treated cells (Figure 9B, left-hand panel). In contrast, when anti-Sp3 antibody was added to the system, the intensity of remaining band 1 (Sp1 or Sp1-like) was enhanced in the nuclear extract from Ang-II-treated cells (Figure 9B, right-hand panel). There was a 2.99 ± 0.38 -fold increase in the density of the remaining band 1 in anti-Sp3-antibody-treated nuclear extract from Ang-II-treated cells compared with the non-treated control cells. Thus the element of band 1 that was enhanced by Ang II was supershifted by anti-Sp1 but not by anti-Sp3.

The effect of transfection with D-Sp1-ODN in mesangial cells treated with Ang II was studied. D-Sp1-ODN inhibited the Ang-II-induced PAI-1 mRNA up-regulation in a dose-dependent manner, whereas the mutated control M D-Sp1-ODN had little effect on the induced expression (Figure 10). Densitometric analysis revealed a $68 \pm 9\%$ ($n = 4$) reduction in Ang-II-induced PAI-1 mRNA expression in the cells transfected with 5 μ M D-Sp1-ODN. Mesangial cells not treated with Ang II showed a $20 \pm 6\%$ reduction in PAI-1 mRNA expression when transfected with 5 μ M D-Sp1-ODN. M D-Sp1-ODN had little effect on both Ang-II-treated and non-treated cells. Thus Sp1 or an Sp1-like factor that is supershifted with anti-Sp1 antibody is likely to be involved both in basal and Ang-II-induced PAI-1 mRNA expression.

DISCUSSION

Our previous study demonstrated that Ang II up-regulates PAI-1 mRNA through PKC activation in glomerular mesangial cells [7]. The present study examined the molecular mechanism of Ang-II-induced up-regulation of *PAI-1* gene expression. Up-regulation of the gene expression has been shown to be transcriptionally regulated by its proximal promoter region. The

decoy ODN (D-P-ODN) of the promoter region, which contains two TRE-like sequences and one Sp1 binding site-like sequence, abolished the gene expression induced by Ang II.

The gel-shift assay, gel supershift assay and methylation-interference experiment in the present study suggest a unique protein–DNA interaction of *PAI-1* gene regulation in mesangial cells. These studies demonstrated that general transcription factors, Sp1 and Sp3, or factors that bind to the GT box and cross-react with anti-Sp1 and anti-Sp3 antibodies, bind to the proximal promoter region of the *PAI-1* gene. In the gel-shift assay, anti-Sp1 and anti-Sp3 antibodies substantially supershifted the complex (band 1) of the D-P-ODN and the mesangial-cell nuclear extract. The methylation-interference experiment revealed that the core sequence of the binding site for Sp1 is located in the so-called GT box, which contains the Sp1-box 1. Sp1 and Sp3 have been shown to regulate basal transcription of many genes [9]. In the gel-shift assay, the intensity of the complex, which contains Sp1 or an Sp1-like factor, was significantly enhanced in nuclear extract from Ang-II-treated mesangial cells compared with untreated control cells. In contrast, the intensity of the Sp3 or Sp3-like factor was not significantly different between Ang-II-treated and non-treated cells. The functional significance of the Sp1 and Sp3 binding to the GT box was examined by the use of decoys. The D-Sp1-ODN decoy, which masks the GT box, abolished the Ang-II-induced PAI-1 mRNA up-regulation, and a mutation in the D-Sp1-ODN resulted in a loss of the inhibitory effect of the D-Sp1-ODN. Transcription of the *PAI-1* gene is likely to be regulated by a balance between the activity of Sp1 and Sp3. Sp3 has been shown to repress transcriptional activity of Sp1 [9].

Several mechanisms for the activation of Sp1 or Sp1-like factor are postulated. Ang II has been reported to induce Sp1 protein in neonatal cardiac fibroblasts [11]. However, we did not observe induction of Sp1 protein by Ang II in rat mesangial cells (results not shown). Another mechanism of Sp1 activation is phosphorylation or dephosphorylation of the protein. Sp1 has been reported to be activated by both phosphorylation and dephosphorylation [12–15]. Mitogen-activated protein kinase (MAPK) has been shown to activate Sp1 [16,17]. Ang II has been proposed to stimulate MAPK in a PKC-dependent manner [18,19]. Our previous data demonstrated up-regulation of *PAI-1* gene expression by Ang II through PKC and protein tyrosine kinases downstream of PKC [7]. Taken together, binding of Sp1 to the GT box may be increased by the PKC- and MAPK-dependent signalling pathway in response to Ang II.

It has been reported that the two TRE-like sequences in the proximal promoter region of the *PAI-1* gene mediate signals from the PKC activator, phorbol ester, in MCF-7 and HepG2 cells [8,20]. Thus, we initially anticipated that Ang-II-induced PAI-1 up-regulation was mediated by the two TRE-like sequences. To our surprise, decoy ODNs encoding the two respective TRE-like sequences were not effective in blocking the induction. On the other hand, there was a weak but detectable supershift by anti-AP-1 antibody in the gel shift. Because of a weak affinity of the two TRE-like sequences for AP-1 compared with the TRE-consensus sequence [8], a larger amount of the decoy ODN may have been required to inhibit AP-1. In this regard, a decoy ODN containing an AP-1 consensus sequence showed a weak inhibitory effect (approx. 20%) on Ang-II-induced PAI-1 mRNA up-regulation (results not shown).

Nevertheless, the present study shows that Sp1, or an Sp1-like factor, is the effector of Ang-II-induced up-regulation of the *PAI-1* gene in mesangial cells. Since various decoy ODNs have been used successfully in experimental models of glomerular diseases [10,21], our approach to suppress extracellular matrix

formation by mesangial cells may have clinical implication as a potential molecular pharmacological tool to control progressive renal diseases.

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