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Suppression of Mesangial Cell Proliferation and Extracellular Matrix Production in Streptozotocin-Induced Diabetic Rats by Sp1 Decoy Oligodeoxynucleotide In Vitro and In Vivo

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Abstract Transcription factor Sp-1 is an important fibrogenic factor that is involved in the pathogenesis of diabetic nephropathy. In this study, we examined the effect of Sp1 decoy oligodeoxynucleotides (ODNs) on the extracellular matrix (ECM) gene expression in cultured rat mesangial cells (RMC) and streptozotocin (STZ)-induced diabetic rats. The ring-type Sp1 decoy ODNs significantly decreased ECM mRNA expression and Sp1 binding to the promoter region of these PDGF-induced genes in RMC. In addition, the decoy ODNs was introduced into the left renal artery of diabetic rat using the hemagglutinating virus of Japan (HVJ)-liposome mediated gene transfer method and effectively delivered to the kidney. On 14 days after ring-type Sp1 decoy ODNs injection, type IV collagen, fibronectin mRNA, and protein expression were markedly decreased, and the rate of urinary creatinine excretion was reduced in the ring-type Sp1 decoy ODNs-treated diabetic rats. These results indicated that the ring-type Sp1 decoy ODNs would be superior to P-Sp1 ODNs. Also, the R-Sp1 decoy ODN when introduced in vivo, effectively reduced ECM production during the progression of nephropathy. Therefore, ring-type Sp1 decoy is a promising tool for developing new therapeutic applications for progressive diabetic nephropathy. J. Cell. Biochem. 103: 663–674, 2008.

Key words: Sp1 decoy oligodeoxynucleotides; mesangial cell; extracellular matrix; streptozotocin; diabetic nephropathy

Diabetic nephropathy is a serious complication in diabetic subjects and is a leading cause of end-stage renal disease and mortality. The characteristic morphological and ultra structural changes in patients with diabetic nephropathy are expansion of the mesangial matrix. Mesangial expansion, caused by the proliferation of MC and the excessive accumulation of extracellular matrix (ECM) proteins, is one of the pathologic features of glomerular disease associated with diabetes mellitus [Steffes et al., 1989]. While mesangial expansion in renal disease, platelet-derived growth factor (PDGF) plays an important role in the production of ECM proteins [Waldherr et al., 1993].

PDGF is a polypeptide that was originally purified from human platelets as a potent mitogen for fibroblasts, osteoblasts, smooth muscle, and mesangial cells [Heldin and Westermark, 1990]. Previous studies have indicated that PDGF is a powerful factor for mesangial cell proliferation [Shultz et al., 1988; Gilbert et al., 2001]. Recently, it has been reported that PDGF plays an important role not only in cell proliferation but also in the synthesis of ECM protein, an increased expression of pro-sclerotic cytokines

Abbreviation used: ECM, extracellular matrix; FN, fibronectin; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; M-Sp1 decoy, mutated Sp1 decoy; ODN, oligodeoxynucleotide; P-Sp1 decoy, phosphorothioate Sp1 decoy; α-SMA, α-smooth muscle actin; R-Sp1 decoy, ring Sp1 decoy; PDGF, platelet-derived growth factor; MC, mesangial cell; EMSA, electrophoretic mobility shift assay; STZ, streptozotocin.

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and growth factors [Ross, 1989; Floege et al., 1993; Isaka et al., 1993]. Magae et al. [1988] reported that the mRNA expression of PDGF was significantly increased in the glomeruli of patients with mesangial proliferate glomerulonephritis compared with normal glomeruli. Therefore, the specific inhibition of PDGF action is a major therapeutic target in the treatment of glomerular disease.

Sp1 regulates the expression of a wide variety of genes, including TGF-β1, fibrogenic cytokine, and a wide variety of matrix genes [Irvine et al., 2005]. Furthermore, Sp1 factors are involved in many growth-related signal transduction pathways including apoptosis, and angiogenesis, and profibrogenesis [Holmgren et al., 1995]. Sp1 is a family of ubiquitous transcription factors that are largely associated with GC-rich promoters, and are mainly involved in basal promoter activity [Cook et al., 1999]. Sp1 exists in a variety of isoforms which bind with varying affinities to sequences designated as Sp1 sites, making up a transcriptional network that plays an important role in the fine tuning of gene expression. Sp1-dependent transcription is altered during cell growth through gene expression [Goldberg et al., 2000].

Decoy technology has recently been developed in an attempt to reduce the activity of a specific transcription factor through the use of a synthetic double-stranded oligodeoxynucleotide (ODN) containing the consensus binding sequence of a transcription factor [Magae et al., 1993]. It has been proposed as a novel therapeutic tool for use in molecular medicine for the treatment of several disorders [Magae et al., 1993]. However, in the period of early gene therapy, a major problem is the rapid degradation of phosphodiester ODNs by nuclease both in vitro and in vivo. To circumvent this problem, various modified DNA analogs, particularly ODNs with phosphorothioate-(PS) modified ODNs, oxygen atoms in the phosphodiester bond of the terminal nucleotides are replace with sulfur atoms. This structural modification increases resistance to nuclease attack [Bielinska et al., 1990; Park et al., 1999]. In this study, we used the R-Sp1 decoy ODN in order to enhance the stability of the decoy.

In the present study, we report that the R-Sp1 decoy ODN blocks the production of ECM and its downstream target genes in cultured rat mesangial cells (RMC) and diabetic nephropathy [Chae et al., 2006]. The findings also show that the R-Sp1 decoy ODN when introduced in vivo, effectively reduced ECM production during the progression of nephropathy.

MATERIALS AND METHODS

Isolation of Rat Glomeruli and Primary Mesangial Cell Culture

Primary RMC were isolated, and cultured as previously described [Park et al., 2003]. RMC were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 20% FBS for 4 days and the medium was then changed every other day until confluence was attained. All assays were performed on cells at passages three to seven. Near confluent cells were incubated with serum-free medium for 24 h to arrest and synchronize the cell growth phase. The medium was then replaced with fresh serum-free medium containing 10 ng/ml of PDGF-BB, and cells were incubated for up to 48 h. In vitro gene transfection of Sp1 decoy ODNs were administered before the addition of PDGF. The characterization of RMC was confirmed by morphology and by an immunohistochemical staining method using an anti-vimentin antibody (DAKO Japan Co., Kyoto, Japan) and an anti-cytokeratin antibody (DAKO Japan Co.).

Construction of Ring-Type Sp1 Decoy ODN

The sequences of Sp1 decoy ODN including phosphorothioated double-stranded ODN (P-Sp1 decoy), ring Sp1 decoy ODN (R-Sp1 decoy) and mutated Sp1 decoy ODN (M-Sp1 decoy) were as follows: P-Sp1 decoy (note: consensus sequences are underlined), 5′-A_STTAC<u>GGGGCGGGGC</u>GAAT$_S$C$_S$-3′; R-Sp1 decoy ODN, 5′-GCCCCGATCTTTTGATC<u>GGGGCGGGGC</u>GAGCTTTTGCTCGCCCC-3′, M-Sp1 decoy ODN, 5′-GTACCGATCTTTTGATC<u>GGTACGGTAC</u>GAGCTTTTGCT CGTACC-3′. ODNs were annealed for 2 h at a decreasing temperature (80–25°C). Following the addition of T4 DNA ligase (1 U), the mixture was incubated for 24 h at 16°C to generate a covalently ligated ring-type decoy molecule. The R-Sp1 decoy ODN was predicted to form a stem-loop structure.

[^{3}H]-Thymidine Incorporation

One μCi/ml of [^{3}H]-thymidine (Du Pont Co., Wilmington, DE) was added to each well 12 h prior to the end of the experimental periods. The cells were then washed twice with phosphate

buffered saline (PBS) and tryPinized before harvesting with a cell harvester (Titertek Cell Harvester 550, Flow Laboratories, Irvine, Scotland, UK). They were placed in a 3 ml scintillation cocktail solution, and the radioactivity was measured by using a β-counter (TL 5000s, Beckman instruments, Inc., Fullerton, CA).

To Determine the Transfection Efficiency

RMCs were seeded with fresh culture medium the day before decoy was added and washed twice with Opti-MEM (Gibco BRL), prior to the experiment. Next, cells were transfected with 0.5 μg of fluorescein-labeled-Sp1 decoy ODN combined with LipofectAMINE Plus™ (Invitrogen, Carlsbad, CA) at 37°C for 24 h. Sp1 decoy ODN was labeled using the Label IT Fluorescein Nucleic Acid Labeling Kit (Panvera Corp., Madison, WI). The RMCs were then washed three times with PBS and fixed in methanol at 4°C for 10 min. Fixed cells were incubated for 30 min at room temperature with Hoechst 33258 (50 ng/ml) and then washed with PBS. The cells were mounted and observed by fluorescence microscopy.

Electrophoretic Mobility Shift Assay (EMSA)

In a typical experiment, DNA probes labeled with [γ-^{32}P]ATP (Amersham, Little Chalfont, UK) and T4 polynucleotide kinase (Promega, Medison, WI) were incubated with 6 μg of nuclear extract, 100 μg/ml poly dI:dC, 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM $MgCl_2$, and 4% glycerol. Labeled ODNs were purified using a NAP-5 column (Phamacia, Uppsala, Sweden). Protein-DNA-binding reactions were performed at room temperature for 20 min. After the incubation, samples were loaded on 4% native polyacrylamide gels with 0.5 × TBE running buffer (45 mM Tris, 45 mM boric acid, and 1 mM EDTA) and run at 150 V for 2 h. Gels were dried and visualized by autoradiography. In some experiments, a 50- to 100-fold molar excess of cold competitor was added to the reaction mixture before adding the nuclear extracts.

Luciferase Assay

Cells were fed with fresh culture medium for 24 h, washed twice with Opti-MEM (Gibco) prior to the experiment, and transfected with 100 nM of Sp1 decoy ODN combined with Lipofectamine Plus™ reagent (molar ratio; DNA:lipid = 1:3) (Gibco). The decoy ODN:lipid mixture was added dropwise to cells, according to the manufacturer's instructions. The luciferase-reporter plasmid, Sp1-luc was a generous gift from Dr. Peggy J. Farnham (University of Wisconsin, USA). pGL3-luc (fibronectin promoter construct) and p3TP-luc (TGF-β1 promoter construct) has been described previously [Chae et al., 2006]. To analyze luciferase expression, the cells were washed twice with PBS and lysed in 200 μl of 1 × reporter lysis buffer (Promega). Each lysate (50 μl) was examined for luciferase activity by means of a commercial kit (Promega).

Cell Lysate, Protein Quantitation, and Western Blotting

As previously study [Kang et al., 2006], Harvested cells were mixed with lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% NP40, 100 mM PMSF, leupeptin 1 mg/ml, apronitin 1 mg/ml, 1 mM DTT). Protein concentrations were determined with commercial Bradford reagent (Bio-Rad, Hercules, CA), and separated by SDS-PAGE. Proteins were then transferred to Protran nitrocellulose transfer membrane (Schleicher & Schuell BioScience, Inc., Germany) and analyzed using antibodies raised against TGF-β1 (1:2,000), type IV collagen (1:2,000), laminin (1:500), fibronectin (1:5,000), and β-actin (all at 1:2,000). After, the membrane was incubated with the appropriate secondary antibody (1 h at room temperature) and the proteins were visualized with an enhanced chemiluminescence (ECL) detection system (Amersham Bioscience).

RNA Preparation and Northern Blot Analysis

Total RNA was isolated from the cells using TRIzol reagent (Sigma, Louis, MO). For Northern analysis, 20 μg aliquots of total RNA were separated on 1% agarose-formaldehyde gels, transferred to nylon membranes (Hybond-N+; Amersham), and cross-linked by ultraviolet irradiation. Partial clones of rat TGF-β1 cDNA, type IV collagen cDNA, laminin cDNA, fibronectin cDNA, and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were labeled by the random primer labeling method with [α-^{32}P]dATP, using a random primer labeling kit (Amersham). After, the labeling reaction, the radiolabeled probes were purified on an NAP-5 column. Hybridization was carried out at 42°C overnight in 50% formamide, 10 × Denhardt's solution, 1% SDS, 5 × standard

saline citrate (SSC), 50 mM sodium phosphate, and 200 μg/ml salmon sperm DNA. The blots were washed three times at 50°C in 0.5 × SSC with 0.1% SDS and blots were exposed to X-ray films at −70°C with an intensifying screen for the appropriate time period. The densities of bands were measured by the Quantity One 1D image analysis software program (Bio-Rad). Also, we used GAPDH as an internal control to standardize the amount of total RNA utilized for Northern blot analysis.

Histology and Immunohistochemisty

For the immunohistochemistry, sections were incubated with anti-type IV collagen (Santa Cruz Biotechnology), fibronectin (Santa Cruz), and α-smooth muscle actin (α-SMA) antibody (DAKO Japan Co.), and were processed for immunohistochemistry in the standard manner. After incubation with a biotin-conjugated secondary antibody, the specimens were processed using an LSAB+ kit (DAKO Japan Co.) and developed with 3,3′-diaminobenzidine tetrahydrochloride.

Preparation of HVJ-Liposome

The hemagglutinating virus of Japan (HVJ)-liposomes were prepared as described previously [Ahn et al., 2002] with minor modifications. HVJ was grown in chorioallantoic fluid of 10-day-old embryonated chicken eggs at 35.5°C. HVJ was collected by centrifugation at 3,000 rpm for 10 min and suspended in balanced salt solution (BSS; 140 mM NaCl, 5.4 mM KCl, 10 mM Tris–HCl, pH 7.5). HVJ was purified by a second centrifugation at 12,000 rpm for 1 h, resuspended in BSS, and stored at 4°C until used. Egg yolk phosphatidyl choline (ePC; Sigma), dioleoyl phosphatidyl ethanolamine (DOPE; Avanti Polar Lipid, Birmingham, AL), egg yolk sphingomyelin (eSph; Sigma), bovine brain phosphatidyl serine (bP; Sigma), and cholesterol (Chol; Sigma) were each dissolved in chloroform, mixed in a weight ratio of 1.6:3:1.5:1.3:1.5, and then dried on a rotary evaporator. Dried lipid was hydrated in 200 ml balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, 10 mM Tris–HCl, pH 7.6) containing ODN. Liposomes were prepared by shaking and filtration. Purified HVJ (Z strain) was inactivated by UV irradiation for 3 min immediately before use. The liposome suspension was mixed with HVJ in a total volume of 2 ml BSS. The mixture was incubated at 41°C for 5 min, followed by 30 min incubation with gentle shaking at 37.1°C. Free HVJ was removed from HVJ liposomes by sucrose density gradient centrifugation. The top layer of the sucrose gradient was collected for use.

Experimental Diabetic Animal Model and In Vivo Transfer of ODNs

Diabetes was induced in 9–10 weeks old male SD rats (180–210 g) by two consecutive intraperitoneal injections of streptozotocin (STZ, Sigma) in 10 mM sodium citrate buffer (pH 4) at 55 mg/kg body wt. Following detection of high blood glucose (>250 mg/dl), rats were anesthetized with sodium pentobarbital (50 mg/kg body weight), and the left renal artery of each animal was surgically exposed. A cannula was introduced into the left renal artery via the aorta. The proximal segment of the renal artery was transiently ligated and HVJ-liposome complex was infused into the renal artery over 5 min. Upon completion, the infusion cannula was removed and blood flow to the kidney was restored by release of ligatures, and the wound was then closed. Each group was sacrificed at 1, 7, and 14 days after injection of ODNs. Normal rats were sacrificed on 14 days. Rats were anesthetized with ethyl ether and the kidneys were removed, immediately frozen in liquid nitrogen, and stored at −70°C for subsequent RNA extraction. Portions of tissues were fixed in neutral buffered formalin for immunohistochemical staining. At the end of each experimental period, individually collected urine was centrifuged at 3,000 rpm at 4°C for 10 min. The supernatant was collected and stored at −70°C until used.

Plasma Albumin and Urine Creatinine Measurement

Urine from each animal was used for the determination of creatinine and albumin. Urine albumin concentration was measured by rat albumin enzyme immunoassay as per the manufacturer's instructions (Cayman). Creatinine was measured using a colorimetric method, based on the Jaffe reaction, as per the manufacturer's instructions (Sigma). The ratio of urinary albumin to creatinine was determined from the measured values.

Statistical Analyses

All experimental results and measurements are expressed as the mean ± standard deviation

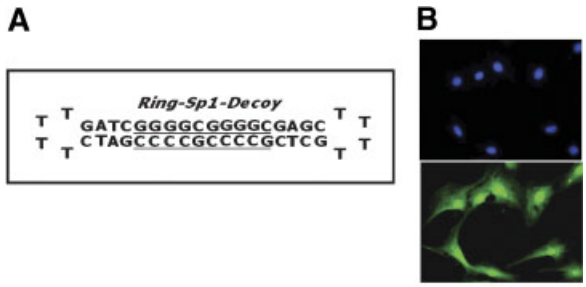

Fig. 1. Structure and transfection efficiency of the R-Sp1 decoy ODN. **A**: The structure of R-Sp1 decoy ODNs. **B**: Fluorescence microscopy of rat MC transfected with Sp1 decoy ODNs in cationic liposomes. After being fixed, the cells were stained for the nuclear marker Hoechst 33258 (**upper panel**). Fluorescein-labeled-Sp1 decoy ODNs were observed in both the nuclei and the cytoplasm (400×) (**lower panel**). Fluorescence levels of Sp-1 decoy ODN were determined with a spectrofluorophotometer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(SD), and statistical comparisons were done using ANOVA followed by the Turkey method for the multiple comparison. *P*-values below 0.05 were considered to be statistically significant.

RESULTS

The Transfection Efficiency of Ring-Type Sp1 Decoy ODN In Vitro

In previously study, we showed that the molecular stability of ring-type Sp1 decoy ODN in the presence of Sp1 nuclease, exonuclease III, or human or fetal bovine serum [Chae et al., 2006]. Therefore, we checked the transfection efficiency of the Sp1 decoy ODN in RMC. Cells were transfected with FITC-labeled Sp1 ODN combined with the Lipofectamine Plus™ reagent and then examined by fluorescence microscopy. As shown in Figure 1, we counterstained with Hoechst reagent 33258 in order to validate the nuclear localization of any ODN and FITC-labeled ODNs were detected in both the nuclei and the cytoplasm of cells. The transfection efficiency of Sp-1 decoy was $85 \pm 5\%$ in RMC.

Effect of Ring-Type Sp1 Decoy ODN on PDGF-Induced Cell Proliferation

The anti-proliferative effect of the Sp1 decoy ODNs was evaluated using PDGF-induced RMC. After being stimulated by PDGF at a concentration of 10 ng/ml, MC proliferated to $185{,}400 \pm 4{,}560$ cells/well; and R-Sp1 decoy ODNs markedly inhibited the MC proliferation ($97{,}200 \pm 2{,}500$ cells/well) (Fig. 2A). Furthermore, the R-Sp1 decoy ODNs showed a more inhibitory effect than the P-Sp1 decoy ODNs (Fig. 2B).

Reduction in ECM Accumulation by Sp1 Decoy ODN on PDGF-Induced ECM Production

The effect of the R-Sp1 decoy ODN on the endogenous expression of ECM genes in cultured RMC induced by 10 ng/ml PDGF for 48 h

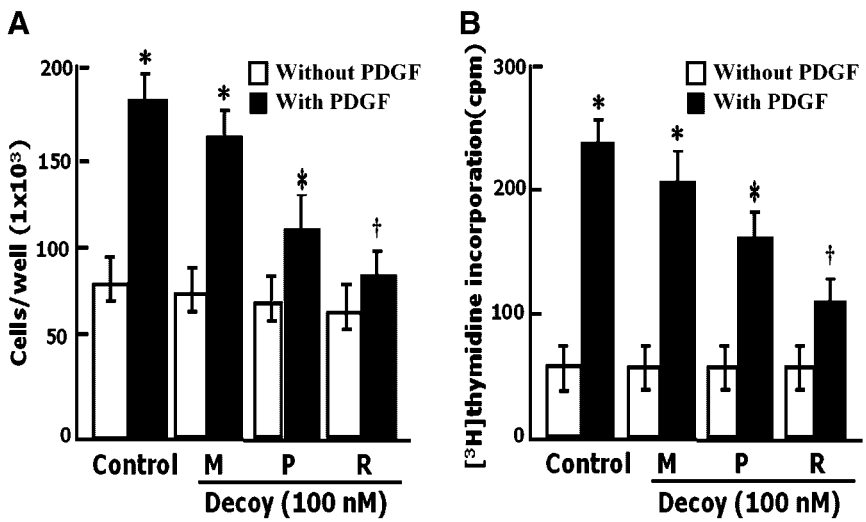

Fig. 2. Effect of Sp1 decoy ODN on PDGF-induced MC proliferation and DNA synthesis. RMC were incubated with the 100 nM Sp1 decoy ODN and PDGF, and were then activated by treatment with PDGF. Cell proliferation was measured by direct cell count (**A**) or by [^{3}H]-thymidine incorporation (**B**). The experimental protocol is detailed in Materials and Methods Section. Data are presented as means ± SD of five experiments. M indicates mutated Sp1 decoy ODN, P indicates posphorothioated Sp1 decoy ODN, and R indicates ring Sp1 decoy ODN. $^*P < 0.05$ versus sp1 decoy ODN without PDGF, $^\dagger P < 0.05$ versus Sp1 decoy ODN with PDGF.

was investigated by Western blot and Northern blot analysis. As shown in Figure 3A, the PDGF-induced protein on MC proliferation and ECM protein synthesis, TGF-β1, type IV collagen, laminin, and fibronectin in RMC were reduced significantly by the R-Sp1 decoy ODN, but not with the M-Sp1 ODN. The effect of the R-Sp1 decoy ODN on the endogenous mRNA expression of TGF-β1, type IV collagen, laminin, and fibronectin in RMC was examined by Northern blot (Fig. 3B). PDGF stimulation significantly increased the endogenous transcription of TGF-β1, type IV collagen, laminin, and fibronectin in RMC, but these genes were decreased by R-Sp1 decoy ODN compared with P-Sp1 decoy ODN and M-Sp1 decoy ODN. These results showed that R-Sp 1 decoy ODN significantly attenuated PDGF-induced expression of these genes.

The effects of the Sp1 decoy ODN on promoter activity were investigated using four reporter plasmids that were introduced into RMC. We initially assessed the effect of the Sp1-decoy ODN on the promoter activity of a reporter gene plasmid, Sp1-luc, which contains six tandem

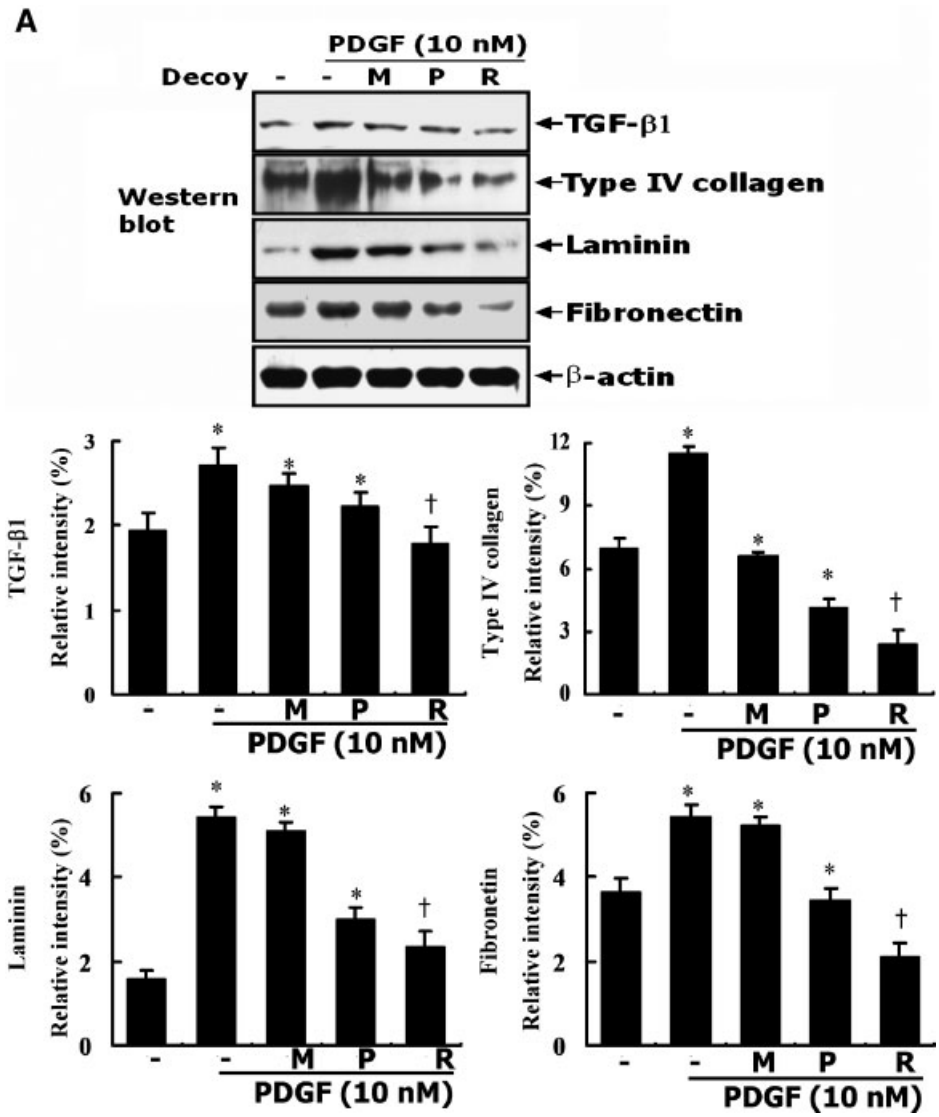

Fig. 3. Effect of Sp1 decoy ODN on PDGF-induced ECM accumulation. RMC transfected with Sp1-decoy ODNs were cultured in medium containing PDGF-stimulation for 24 h. **A**: ECM secretion into the medium was measured by Western blot analysis. **B**: Total RNA was extracted from RMC 1 day after transfection with Sp1 decoy ODN. mRNA expression of ECM production was determined by Northern blotting. Signal intensity was quantified by densitometric analysis. Values represent the mean ± SE of five independent experiments. M indicates mutated Sp1 decoy ODN, P indicates posphorothioated Sp1 decoy ODN, and R indicates ring Sp1 decoy ODN. Statistical significance was determined as $^{*}P < 0.05$ compared to PDGF without Sp1 decoy ODN, $^{\dagger}P < 0.01$ compared to PDGF without Sp1 decoy ODN.

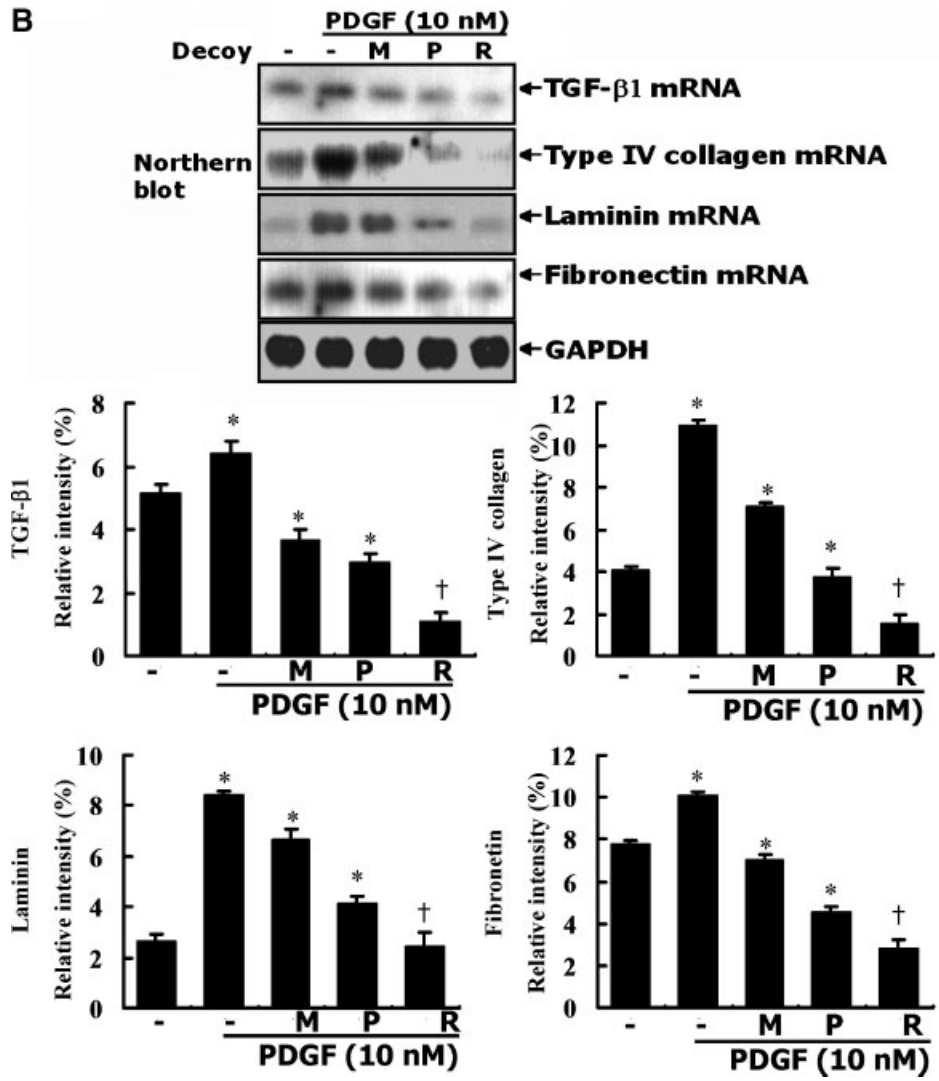

Fig. 3. (*Continued*)

copies of the Sp1-binding sites in the promoter (Fig. 4A). As expected, co-transfection with the R-Sp1 decoy ODN significantly attenuated the enhanced luciferase gene expression induced by PDGF, while the M-Sp1 decoy ODN had a negligible effect on luciferase activity. Similar inhibitory effects of Sp-1 decoy ODNs on promoter activity were observed in experiments in which reporter plasmids involved in MC proliferation and ECM production, TGF-β1 (p3TP-luc, Fig. 4B), type IV collagen (COLL4-α1-luc, Fig. 4C), and fibronectin were used (FN-luc, Fig. 4D).

Effects of Sp1-decoy ODN on the PDGF-Induced DNA Binding Activity of Sp1

EMSA was performed on nuclear extracts of RMC that had been transfected with the Sp1 decoy ODN to determine the effect of the decoy on the PDGF-induced DNA binding activity of Sp1 (Fig. 5A). The binding activity was quantified by a phospho-image analyzer (Fig. 5B). Nuclear cell extracts from cultured RMC that had been treated with PDGF showed a significant increase in DNA binding activity to the consensus sequence for Sp1. The binding was specific, as evidenced by the complete suppression of the formation of the DNA–protein complexes in the presence of an excess of unlabeled competitor ODN. As expected, transfection of the R-Sp1 decoy ODN significantly attenuated the DNA binding activity induced by PDGF-stimulation, whereas the M-Sp1 decoy ODN did not affect the activity induced by PDGF-stimulation. The P-Sp1 decoy ODN was less effective than R-Sp1 decoy ODN.

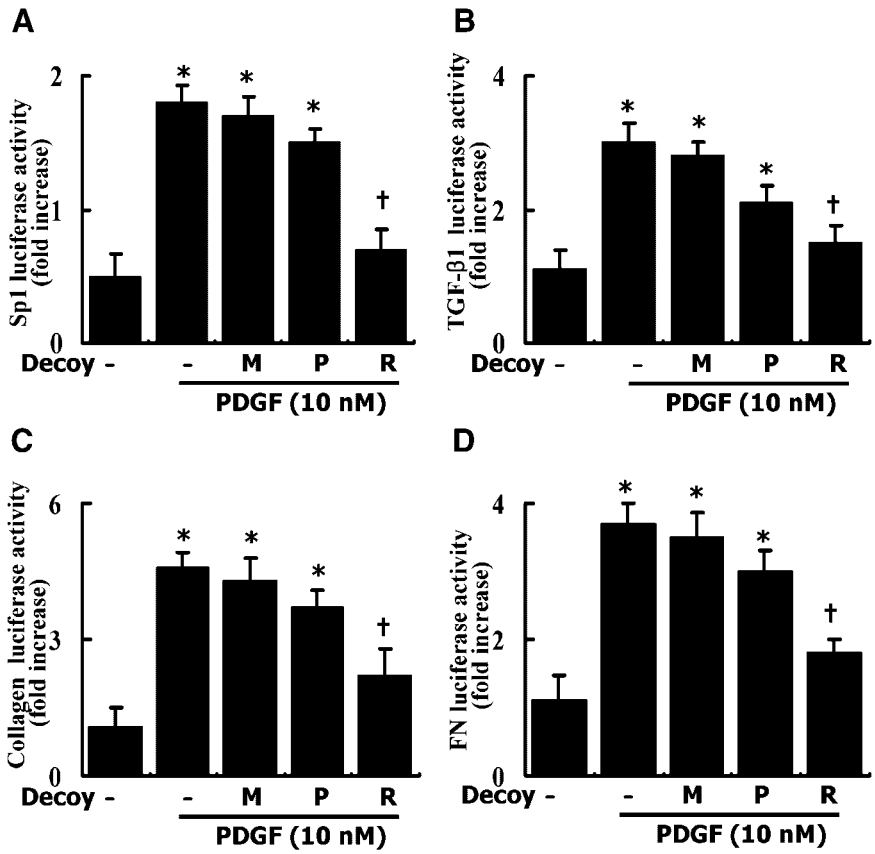

Fig. 4. Sp1 decoy ODN inhibits the Sp1, TGF-β1, type IV collagen, and fibronectin in promoter assay. RMC cotransfected with decoy ODN and Sp1-luc (**A**, positive control), 3TP-lux (**B**, TGF-β1 promoter), COL4α1-luc (**C**, type IV collagen promoter), and FN-luc (**D**, fibronectin promoter) were cultured in medium containing PDGF-stimulated with 100 nM Sp1 decoy ODN for 24 h. Luciferase activity in the cell lysate was determined. Data are presented as the mean ± SE of five independent experiments. Values represent the mean ± SE of five independent experiments. M indicates mutated Sp1 decoy ODN, P indicates posphorothioated Sp1 decoy ODN, and R indicates ring Sp1 decoy ODN. Statistical significance was determined as $^{*}P < 0.05$ compared to PDGF without Sp1 decoy ODN, $^{\dagger}P < 0.01$ compared to PDGF without Sp1 decoy ODN.

The incubation of nuclear extracts with an anti-Sp1 antibody resulted in a supershift of the complex with a concomitant diminution of the original band.

Effects of R-Sp1 Decoy on Type IV Collagen, Fibronectin, α-SMA mRNA, and Protein Expression in STZ-Induced Diabetic Rats

The effect of the R-Sp1 decoy ODNs on mRNA and protein expression of type IV collagen, fibronectin, and α-SMA in diabetic kidneys was determined by RT-PCR, Western blotting, and immunohistochemical study. Sp1-decoy ODNs-treated and untreated diabetic rats were examined 14 days after introduction of the ODNs. Protein expressions of fibronectin, α-SMA, and type IV collagen in the diabetic kidneys were high expression compare with the normal kidneys (Fig. 6A). They were inhibited by R-Sp1 decoy ODNs. Consistent with the results of Western blotting, mRNAs of fibronectin, α-SMA, and type IV collagen were increased in diabetic rats compared to normal ones, and all their expressions were significantly decreased in R-Sp1-ODNs treated diabetic rats compared with untreated diabetic rats (Fig. 6B). Also, R-Sp1 decoy ODNs effectively decreased the urine albumin-to-creatinine ratio (Fig. 6C). In this animal model, no difference was found between the Sp1 decoy ODNs-treated and untreated animal group in blood pressure (106 ± 1.9 ($n = 10$) vs. 109 ± 1.8 ($n = 10$) mm Hg, respectively) or in blood glucose (645 ± 39 ($n = 10$) vs. 659 ± 72 ($n = 10$) mg/dl, respectively).

In an immunohistochemical study of rat kidney tissues after STZ stimulation, the expressions of type IV collagen, fibronectin, and α-SMA were analyzed for untreated kidney tissues compared with M-Sp1, P-Sp1 and R-Sp1

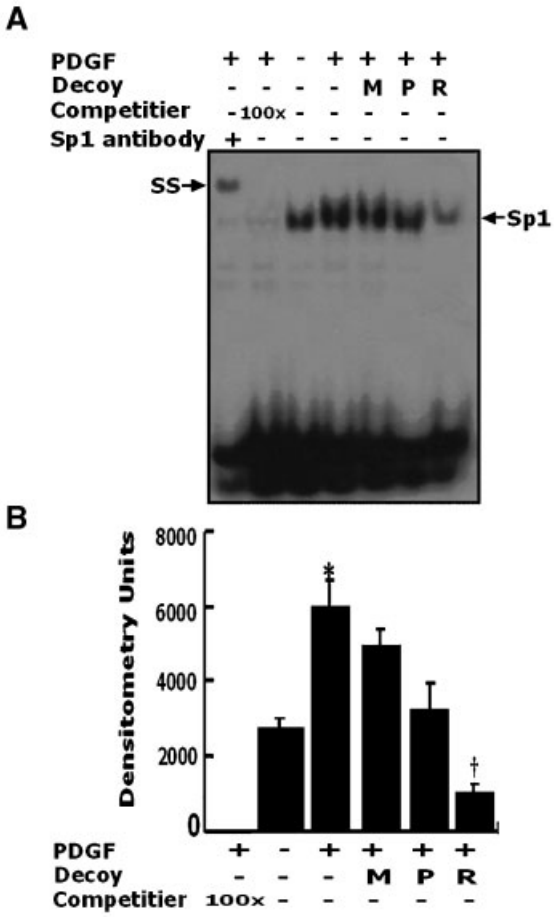

Fig. 5. Sp1 decoy ODN blocks the PDGF-induced DNA binding activity of Sp1. RMC were transfected with decoy ODNs. PDGF was added 24 h after the transfection. Sp1 decoy ODN-treated RMC were harvested 24 h after transfection. Nuclear extracts were prepared and 6 μg of each extract was subjected to EMSA. Typical gel shift assay result for RMC transfected with Sp1 decoy ODNs (**A**) and signal intensity quantified by densitometric analysis (**B**) were shown. M indicates mutated Sp1 decoy ODN, P indicates posphorothioated Sp1 decoy ODN, and R indicates ring Sp1 decoy ODN. Values represent the mean ± SE of five independent experiments. Statistical significance was determined as $^{*}P < 0.05$ compared to PDGF without Sp1 decoy ODN, $^{\dagger}P < 0.01$ compared to PDGF without Sp1 decoy ODN.

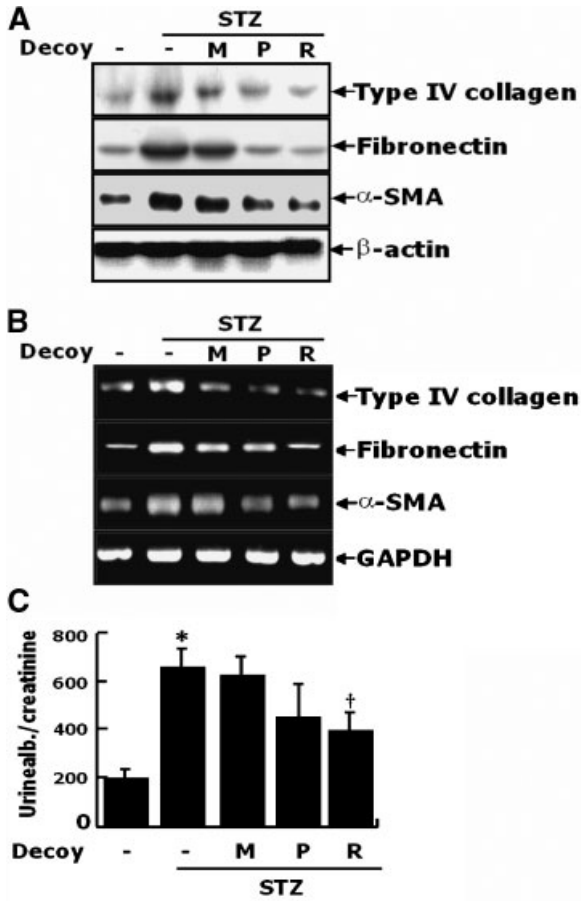

Fig. 6. Effect of Sp1 decoy ODN on in vivo expression of ECM protein and mRNA. **A**: Protein expression of type IV collagen, fibronectin and α-SMA in STZ-induced kidney was measured by Western blot analysis. **B**: Effect of Sp1-decoy ODNs on type IV collagen, fibronectin, and α-SMA mRNA expression in diabetic kidneys 14 days after the injection of ODNs into STZ-diabetic rats was measured by RT-PCR analysis. **C**: Urine albumin-to-creatinine ratio was determined on urine collection 14 days after the transfection (10 rats per group). Values represent the mean ± SE of five independent experiments. Statistical significance was determined as $^{*}P < 0.05$ compared to STZ-diabetic rats without R-ODN, $^{\dagger}P < 0.01$ compared to STZ-diabetic rats without R-ODN.

decoy ODNs treated kidney tissues. Also, we showed the influence of the R-Sp1 ODN on the gene α-SMA gene expression pattern at various time-points (3, 7, and 14 days) (Fig. 7). Protein expressions of α-SMA in the diabetic kidneys were high expression compare with the normal kidneys, while α-SMA was inhibited by R-Sp1 decoy ODNs.

As shown in Figure 8, protein expression levels of type IV collagen (D), fibronectin (I), and α-SMA (N) in diabetic kidneys were reduced by the R-Sp1 decoy ODNs. A computational analysis of histological staining indicated that the reduction, of these ECM proteins by R-Sp1 decoy ODNs was about 50% compared to untreated group (Fig. 8E,J,O). The P-Sp1-decoy ODNs were less effective and the suppressive effect of the M-Sp1 decoy ODN treatment was not statistically significant (Fig. 8E,J,O).

DISCUSSION

Enhanced MC proliferation and a increased level of metabolism of fibronectin, laminin, and

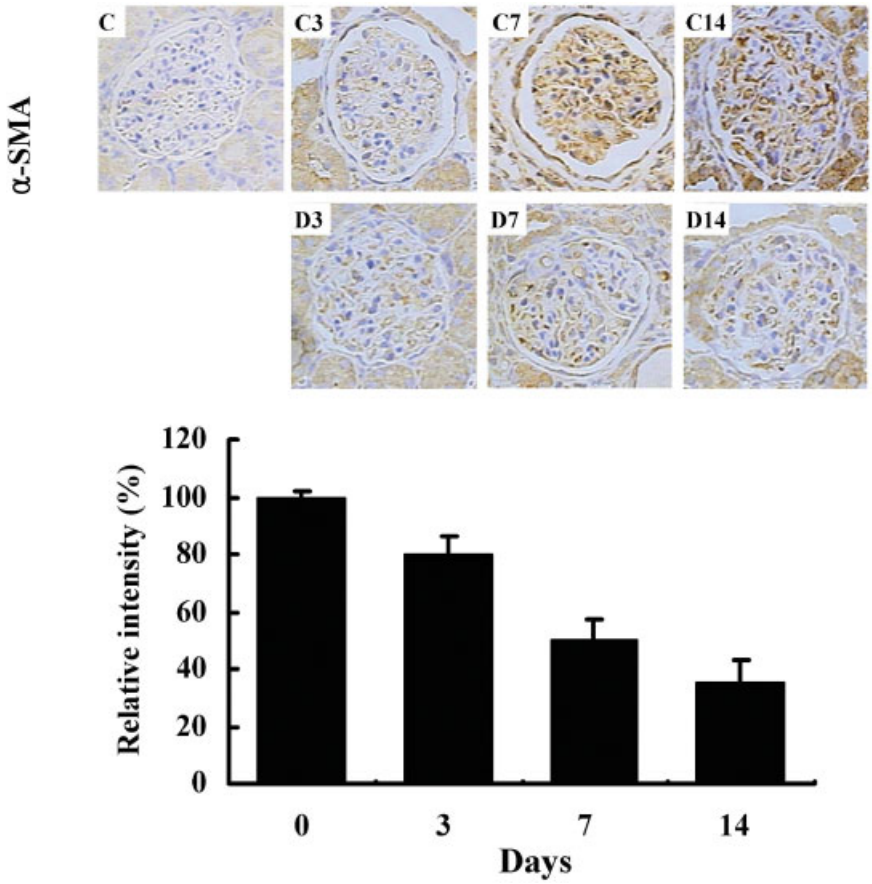

Fig. 7. Effect of R-Sp1 decoy ODNs on expressions of α-smooth muscle actin in STZ-induced diabetic kidney. Immunohistochemistry (IHC) for α-smooth muscle actin was analyzed with STZ-induced diabetic kidney on 3, 7, 14 days after ODNs injection. Photomicrographs are of representative kidney sections with no treatment (**C**), STZ-induced diabetic kidney (**C3**,**C7**,**C14**), R-Sp1 ODNs-treated diabetic kidney (**D3**,**D7**,**D14**). Signal intensity was quantified by densitometric analysis. Values represent the mean ± SE of five independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

collagen IV ultimately restrict glomerular filtration capacity, leading to overt nephropathy, which progresses to end-stage renal disease. PDGF has been consistently implicated in the cell proliferation and ECM accumulation that characterizes progressive glomerular disease [Throckmorton et al., 1995]. Using several complementary approaches, we demonstrated that Sp1 targeting may be a potentially powerful therapeutic approach for reducing ECM accumulation under conditions of diabetes mellitus.

Recently a number of new technologies have been developed to inhibit target gene expression in a sequence-specific manner, and these technologies have been investigated as treatment modalities for a wide variety of diseases. While there are numerous reports on strategies targeting mRNA via antisense oligonucleotides, ribozymes, and RNA interference, targeting proteins that regulate expression of a specific gene is a relatively novel approach. In this method, proteins that regulate the expression of a specific gene are targeted [Bielinska et al., 1990]. This is often achieved by delivering "gene-regulating" molecules such as synthetic double-stranded ODNs that mimic cis-acting promoter elements. Decoy molecules bind to the target transcription factor in a sequence-specific manner and thereby alter gene transcription, producing the desired functional response. Nevertheless, the greatest limitation of these ODNs is that they are easily degraded by nucleases or by readily nonspecific reaction with the control strand. To circumvent theses problems, we have developed a ring-type decoy ODN.

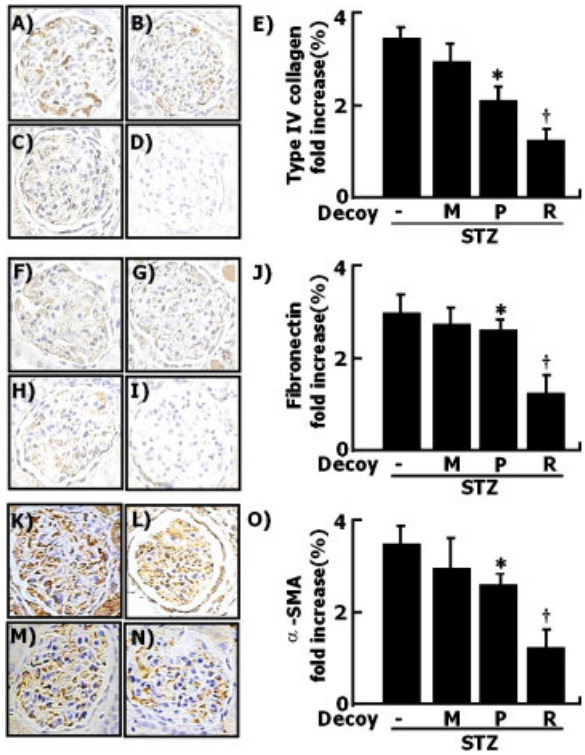

Fig. 8. Effect of Sp1 decoy ODNs on expressions of proteins involved in MC proliferation and ECM protein synthesis. Immunohistochemistry (IHC) for type IV collagen expression (**A**–**D**), fibronectin (**F**–**I**), and α-smooth muscle actin (**K**–**N**) were analyzed with STZ-induced diabetic kidney on 14 days after ODNs injection. Photomicrographs are of representative kidney sections with no treatment (A,F,K), M-Sp1- (B,G,L), P-Sp1- (C,H,M), R-Sp1 ODNs-treated diabetic kidney (D,I,N). Signal intensity was quantified by densitometric analysis (**E**,**J**,**O**) (magnification 200×). Values show independent experiments (n = 10). Statistical significance was determined as $^{*}P < 0.05$ compared to STZ-diabetic rats without Sp1 decoy ODN, $^{\dagger}P < 0.01$ compared to STZ-diabetic rats without Sp1 decoy ODN. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

In this study, we used PDGF-induced RMC activation as an in vitro model of progressive glomerulonephropathy, since the renal expression of PDGF is upregulated in chronic allograft nephropathy and PDGF induces both proliferation and ECM synthesis in MC [Parameswaran et al., 2002]. The R-Sp1 decoy ODN inhibits PDGF-induced rat MC proliferation and downregulated the collagen synthesis. R-Sp1 decoy ODNs at concentrations above 10 nM significantly inhibited the proliferation of PDGF-induced RMC. In addition, the R-Sp1 decoy ODN suppressed the mRNA generation of TGF-β1 and fibronectin as well as cell proliferation induced by PDGF-induced RMC. EMSA showed that the DNA–protein complex induced by PDGF-induction was reduced significantly in RMC that had been transfected with R-Sp1 decoy ODN whereas the M-Sp1 decoy ODN had no effect on DNA binding activity. Experiments using reporter plasmids that contain the Sp1 binding motif in the TGF-β1 and fibronectin promoter demonstrate that the enhancement in Sp1-dependent transcription activity in response to serum stimulation was reduced significantly by the R-Sp1 decoy ODN.

We also demonstrated that in vivo R-Sp1 decoy ODN treatment effectively attenuated the induction of ECM mRNA expression and the resulting deposition of ECM in rat kidney glomerular area of STZ-treated rats. Therefore, these results suggest that the R-Sp1 decoy ODN represents a potentially effective gene therapy strategy to ameliorate mesangial ECM accumulation in STZ-induced nephropathy. Furthermore, R-Sp1 decoy ODNs markedly suppressed the levels of type IV collagen mRNA, fibronectin mRNA and protein in diabetic kidneys. Its specific activity in blocking type IV collagen expression was demonstrated by the absence of any difference in the level of GAPDH mRNA. Various routes for the efficient transfer of ODNs into kidney have been examined in experimental models. Akagi et al. [1996] and Ahn et al. [2004] reported on the transfection of ODNs via the renal artery in an STZ-model.

In conclusion, our data clearly demonstrated that Sp-1 is a key transcription factor mediating mesangial cell proliferation and ECM gene expression involved in preventing the pathogenesis of glomerular disease. However, Sp-1 regulates numerous genes, many of which are not involved in the etiology of diabetic nephropathy. The present study failed to analyze any of these genes and report the disregulation of inappropriate targets. Although there are still many unresolved issues in the clinical application of the ODN-based strategy, the utility of ring-type decoy ODN could be widespread as a useful tool for gene therapy in other diseases. Therefore, this new molecular strategy, using R-Sp1 decoy ODNs with highly effective HVJ-liposome gene-delivery technique, could represent a powerful investigative and potentially therapeutic strategy in the prevention and treatment of diabetic nephropathy.

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