Korean J Physiol Pharmacol
Vol 14: 385−390, December, 2010
DOI: 10.4196/kjpp.2010.14.6.385

Plasminogen Activator Inhibitor-1 Antisense Oligodeoxynucleotides Abrogate Mesangial Fibronectin Accumulation

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Excessive extracellular matrix (ECM) accumulation is the main feature of chronic renal disease including diabetic nephropathy. Plasminogen activator inhibitor (PAI)-1 is known to play an important role in renal ECM accumulation in part through suppression of plasmin generation and matrix metalloproteinase (MMP) activation. The present study examined the effect of PAI-1 antisense oligodeoxynucleotide (ODN) on fibronectin upregulation and plasmin/MMP suppression in primary mesangial cells cultured under high glucose (HG) or transforming growth factor (TGF)-β1, major mediators of diabetic renal ECM accumulation. Growth arrested and synchronized rat primary mesangial cells were transfected with 1 μM phosphorothioate-modified antisense or control mis-match ODN for 24 hours with cationic liposome and then stimulated with 30 mM D-glucose or 2 ng/ml TGF-β1. PAI-1 or fibronectin protein was measured by Western blot analysis. Plasmin activity was determined using a synthetic fluorometric plasmin substrate and MMP-2 activity analyzed using zymography. HG and TGF-β1 significantly increased PAI-1 and fibronectin protein expression as well as decreased plasmin and MMP-2 activity. Transient transfection of mesangial cells with PAI-1 antisense ODN, but not mis-match ODN, effectively reversed basal as well as HG- and TGF-β1-induced suppression of plasmin and MMP-2 activity. Both basal and upregulated fibronectin secretion were also inhibited by PAI-1 antisense ODN. These data confirm that PAI-1 plays an important role in ECM accumulation in diabetic mesangium through suppression of protease activity and suggest that PAI-1 antisense ODN would be an effective therapeutic strategy for prevention of renal fibrosis including diabetic nephropathy.

Key Words: Plasminogen activator inhibitor-1, Antisense oligodeoxynucleotide, Plasmin, Matrix metalloproteinase, Mesangial cells

INTRODUCTION

Excessive accumulation of extracellular matrix (ECM) in the glomerular mesangium is the major pathologic feature in chronic kidney disease including diabetic nephropathy [1]. ECM accumulation results from both increased synthesis and decreased degradation of ECM components [2]. The plasminogen activator (PA)/plasmin/PA inhibitor (PAI) system is thought to play an important role in ECM degradation. Plasmin degrades matrix proteins directly and indirectly through activation of other proteinases such as matrix metalloproteinases (MMP) [3-5].

Plasmin, a serine proteinase, is produced by two well-characterized PAs which are urokinase PA (uPA) and tissue kinase PA (tPA) [6] and this process is regulated by PAI, a specific inhibitor of PAs. PAI-1 is a 50 kD single chain glycoprotein, upregulated by various stimuli including transforming growth factor-β1 (TGF-β1), and activated and stabilized by binding to vitronectin [7-10]. In the normal human kidney, PAI-1 is undetectable in the basal level but the expression is upregulated in several acute and chronic kidney diseases [9,10] including diabetic nephropathy [11]. A functional role of PAI-1 in renal diseases was established mainly by in vivo studies using PAI-1 knock out (KO) mice; renal fibrosis in crescentic glomerulonephritis [12], unilateral ureteral obstruction [13], and diabetic nephropathy [14-16] was attenuated in PAI-1 KO mice. In addition, mutant, non-inhibitory human PAI-1 peptide decreased proteinuria as well as fibrosis in experimental renal injury models [17-19].

Antisense oligodeoxynucleotide (ODN) technique has been developed as a promising strategy to modulate targeted gene deletion in vivo as well as in vitro [20-22]. Antisense ODN has thus become an attractive new therapeutic agent to inhibit disease-related target gene expression, although delivery efficiency of antisense ODN to

Received October 10, 2010, Revised October 30, 2010, Accepted November 6, 2010

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ABBREVIATIONS: HG, high D-glucose; ECM, extracellular matrix; MMP, matrix metalloproteinase; ODN, oligodeoxynucleotides; PAI, plasminogen activator inhibitor; tPA, tissue kinase plasminogen activator; uPA, urokinase plasminogen activator.

target tissue remains to be improved.

Although PAI-1 is known to play an important role in renal ECM accumulation in part through suppression of plasmin generation and MMP activation, the experimental approach for PAI-1 inhibition has been limited in PAI-1 KO mice. The present study, therefore, examined the effect of PAI-1 antisense ODN on fibronectin accumulation and the activity of plasmin and MMP in primary mesangial cells cultured under high glucose (HG) or TGF-β1, which are both major mediators of diabetic renal ECM accumulation.

METHODS

All chemicals and tissue culture plates were obtained from Sigma-Aldrich Company (St. Louis, MO, USA) and Becton Dickinson Labware (Lincoln Park, NJ, USA), respectively, unless otherwise stated.

Mesangial cell culture

Primary rat mesangial cells were isolated from kidneys of 100 to 150 g male Sprague-Dawley rats by a conventional sieving method and characterized as described previously [23]. Cells (between the 8th and 12th passages) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL., Gaithersberg, MD, USA) containing 20% fetal bovine serum (FBS, GIBCO BRL.), 100 U/ml penicillin, 100 μg/ml streptomycin, 44 mM $NaHCO_3$ and 14 mM N-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid (HEPES).

Transfection with PAI-1 antisense ODN

The day before transfection, rat mesangial cells were seeded and the transfection of PAI-1 antisense or mis-match ODN was carried out using Lipofectamine™ Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions with 1 μM phosphorothioate modified ODN (Bioneer, Seoul, Korea). The antisense and mis-match sense ODN sequences were: PAI-1 antisense ODN 5'-GAG GGC TGA AGA CAT C-3', PAI-1 mis-match sense ODN 5'-GAG CGC TGA TGA CAT C-3'. After 24 hour transfection, cells were incubated with serum free media for an additional 24 hours to stop transfection and were then stimulated with 5.6 mM D-glucose, 30 mM D-glucose, or 2 ng/ml of TGF-β1 for 48 hours.

Immunoblot analysis

Immunoblot analysis was performed to determine secreted PAI-1 and fibronectin in the culture media. After measuring the concentration of cellular protein using Bio-Rad protein assay kit, aliquots of media corresponding to the same cellular protein were mixed with sample buffer containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol and heated at 95°C for 5 minutes. Samples were applied to 10% SDS polyacrylamide gel (PAGE) for PAI-1 and 6% SDS PAGE for fibronectin and electrophoresed. After electrophoresis, the proteins were transferred onto nitrocellulose membranes. Membranes were incubated with rabbit anti-rat PAI-1 (American Diagnostica, Greenwich, CT, USA; 1 : 1,000), washed, and incubated with peroxidase-conjugated secondary antibody. For fibronectin, rabbit anti-human fibronectin that combined primary and secondary antibodies (DAKO A/S, Glostrup, Denmark; 1 : 2,000) was used. After washing, the membranes were visualized with enhanced chemiluminescence (Amersham Life Science). Bands were quantified by TINA 2.0 Image program.

Measurement of plasmin activity

Plasmin activity in the culture media was determined as previously described [24] using a synthetic fluorometric plasmin substrate methoxysuccinyl-L-Ala-L-Phe-L-Lys-7-amido-4-methyl-coumarin. 25 μl of 5 fold-concentrated media was mixed with 31.2 μl of dH_2O and 112.5 μl of 0.2 M Tris-HCl, pH 7.4, containing 0.2 M NaCl. Each reaction was initiated by adding 56.3 μl of the substrate in water (final concentration 5.0 μM). Immediately after the addition of substrate, each tube was mixed well, transferred to a 37°C water bath, and incubated for 40 minutes. After the incubation period, each reaction was stopped by the addition of 25 μl of soy bean trypsin inhibitor. The fluorescence of each sample was measured in a fluorometer (Wallac Victor^3 1420 Multilabel Counter, Turku, Finland) at 450 nm emission wave length and at 360 nm excitation wave length, and calculated using a plasmin standard curve.

Measurement of MMP-2 activity

After measuring the concentration of cellular protein using Bio-Rad assay, media corresponding to the same cellular protein were combined with sample buffer containing 0.5 M Tris-HCl, 10% SDS, 0.1% bromophenol blue, and glycerol and incubated 10 minutes at room temperature. Samples were loaded onto 10% SDS PAGE containing 1 mg/ml of gelatin. After electrophoresis, the gels were incubated in the 2.5% (v/v) triton X-100 renaturing buffer for 30 minutes with gentle agitation. Gels were subsequently developed in the buffer containing 50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% (w/v) Triton X-100, pH 7.4. Protein staining was performed with Coomassie brilliant Blue R-250 0.5% (w/v) in 45% (v/v) methanol, 10% (v/v) acetic acid, and destained in the same solution without dye. Digested bands were captured in a gel-scanner and quantified by TINA 2.0 Image program.

Statistical analysis

All results were expressed as means±standard error (SE). Analysis of variance was used to assess the differences between multiple groups. If the F statistics were significant, the mean values obtained from each group were then compared by Fisher's least significant difference. A p value below 0.05 was used to determine statistical significance.

RESULTS

PAI-1 expression levels were suppressed by PAI-1 antisense ODN

In order to confirm the transfection efficiency of PAI-1 antisense ODN under our experimental condition, we analyzed PAI-1 mRNA and protein expression. PAI-1 antisense ODN significantly decreased PAI-1 mRNA (data not shown) and protein (Fig. 1A) expression compared to control. However, PAI-1 mis-match sense ODN did not affect basal expression, confirming selectivity of our antisense ODN to

PAI-1.

Next we examined whether PAI-1 antisense ODN administration had the ability to inhibit PAI-1 upregulation induced in mesangial cells cultured under diabetic conditions. Both 30 mM high D-glucose and 2 ng/ml TGF-β1 significantly increased mesangial cell PAI-1 protein secretion at 48 hours (Fig. 1). PAI-1 antisense ODN effectively inhibited both HG- and TGF-β1-induced PAI-1 protein secretion down to control levels. As expected, PAI-1 mis-match sense ODN did not affect basal or upregulated PAI-1 protein expression (Fig. 1A).

Suppressed plasmin activity was recovered in PAI-1 antisense ODN-transfected mesangial cells

Both HG and TGF-β1 suppressed plasmin activity in primary rat mesangial cells, and these suppressions were recovered by PAI-1 antisense ODN (Fig. 2). PAI-1 antisense ODN also increased basal plasmin activity (Fig. 2), suggesting that PAI-1 suppresses basal plasmin activity in cultured mesangial cells. Neither basal nor HG-suppressed plasmin activity was affected by PAI-1 mis-match sense ODN (Fig. 2A).

MMP-2 activity was restored in PAI-1 antisense ODN-transfected mesangial cells

Suppressed MMP-2 activity was also restored by PAI-1 antisense ODN in HG- and TGF-β1-treated mesangial cells (Fig. 3). Additionally, PAI-1 antisense ODN increased basal MMP-2 activity (Fig. 3), as did basal plasmin activity. PAI-1 mis-match sense ODN did not show any effect on basal or HG-stimulated MMP-2 activity (Fig. 3A).

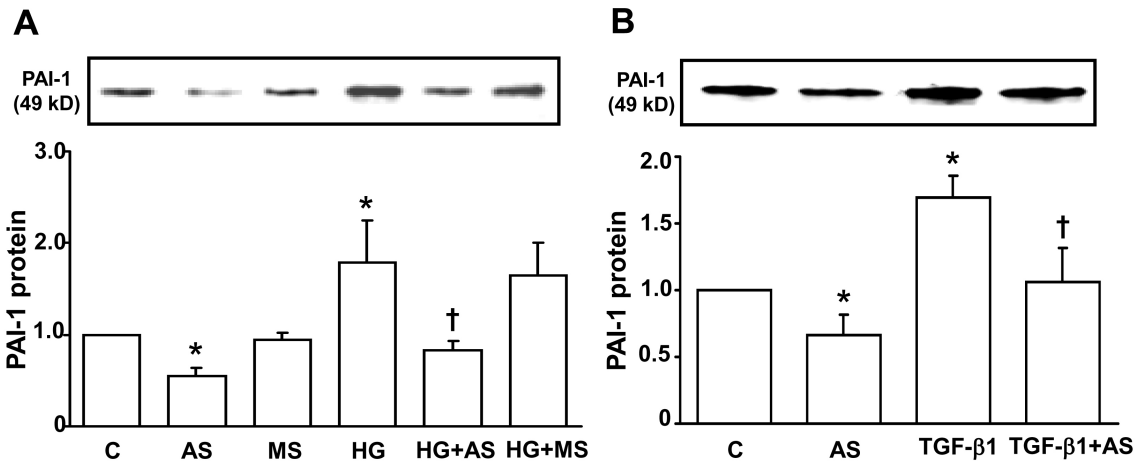

Fig. 1. Effect of PAI-1 antisense ODN on HG- (A) and TGF-β1- (B) induced PAI-1 protein expression in mesangial cells. Growth arrested and synchronized primary rat mesangial cells were stimulated with 30 mM high D-glucose (HG) (A) or 2 ng/ml of TGF-β1 (B) for 48 hours after PAI-1 antisense or mis-match sense ODN transfection. PAI-1 protein was measured by Western blot analysis as described in the Methods. Data are presented as means±SE of 4 experiments. *$p<0.05$ vs control, $^{\dagger}p<0.05$ vs HG or TGF-β1, C: control without HG or TGF-β1, AS: PAI-1 antisense ODN, MS: PAI-1 mis-match sense ODN.

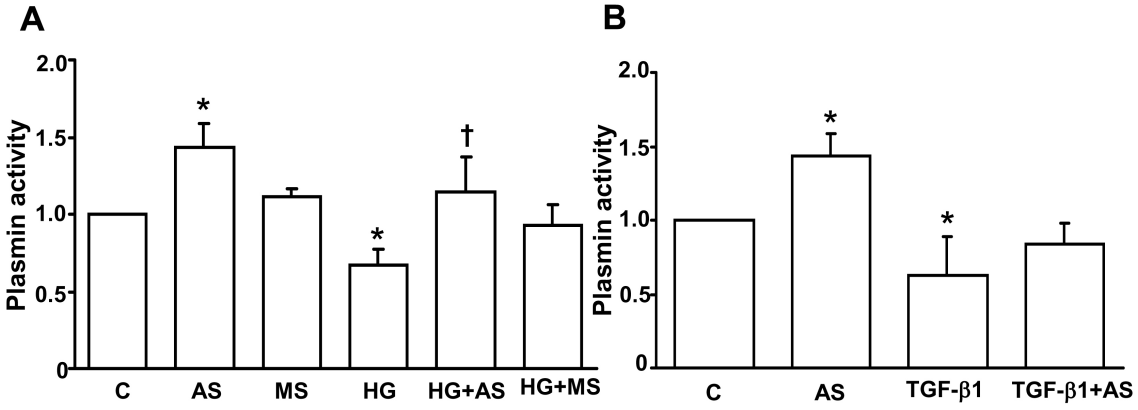

Fig. 2. Effect of PAI-1 antisense ODN on HG- (A) and TGF-β1- (B) suppressed plasmin activity in mesangial cells. Growth arrested and synchronized primary rat mesangial cells were stimulated with 30 mM high D-glucose (HG) (A) or 2 ng/ml of TGF-β1 (B) for 48 hours after PAI-1 antisense or mis-match ODN transfection. Plasmin activity in the media was measured as described in the Methods. Data are presented as means±SE of 4 experiments. *$p<0.05$ vs control without HG or TGF-β1, $^{\dagger}p<0.05$ vs HG or TGF-β1, C: control without HG or TGF-β1, AS: PAI-1 antisense ODN, MS: PAI-1 mis-match sense ODN.

Fibronectin protein upregulation was also abrogated in PAI-1 antisense ODN-transfected mesangial cells

HG and TGF-β1 induced fibronectin secretion, as well as PAI-1 antisense ODN, but not mis-match sense ODN, decreased fibronectin upregulation (Fig. 4). PAI-1 antisense ODN also reduced basal fibronectin protein secretion.

DISCUSSION

The present study demonstrated that suppression of PAI-1 gene by PAI-1 antisense ODN effectively increased plasmin and MMP-2 activity and decreased fibronectin secretion in primary mesangial cells. It is well known that renal PAI-1 expression is overexpressed in pathologic conditions associated with fibrosis including diabetic nephropathy [11]. PAI-1 is also overexpressed in renal cells cultured under diabetic conditions such as HG [25] and TGF-β1 [4,24,26]. Data from genetically modified mice such as PAI-1 overexpressed transgenic or null mice suggest PAI-1 as a therapeutic target for renal fibrosis. With respect to therapeutic interventions for renal PAI-1, mutant non-inhibitory PAI-1 peptide [17-19] and PAI-1 decoy peptide [27] have been used to block PAI-1 synthesis in vivo as well as in vitro. In the present study, phosphorothioate-modified PAI-1 antisense ODN was used to study the role of PAI-1

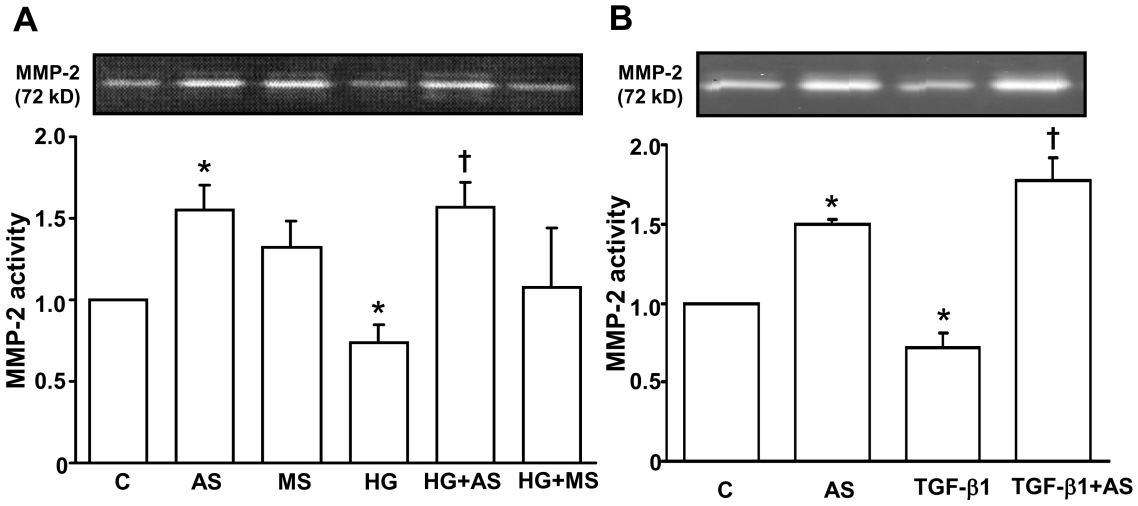

Fig. 3. Effect of PAI-1 antisense ODN on HG- (A) and TGF-β1- (B) suppressed MMP-2 activity in mesangial cells. Growth arrested and synchronized primary rat mesangial cells were stimulated with 30 mM high D-glucose (HG) (A) or 2 ng/ml of TGF-β1 (B) for 48 hours after PAI-1 antisense or mis-match ODN transfection. MMP-2 activity was detected by gelatin zymography as described in the Methods. Data are presented as means±SE of 4 experiments. *$p<0.05$ vs control without HG or TGF-β1, $^{\dagger}p<0.05$ vs HG or TGF-β1, C: control without HG or TGF-β1, AS: PAI-1 antisense ODN, MS: PAI-1 mis-match sense ODN.

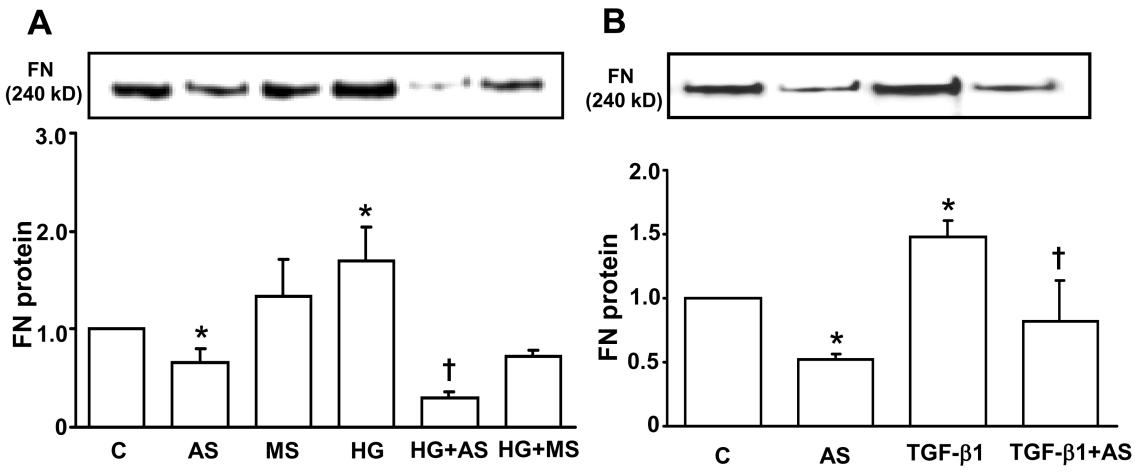

Fig. 4. Effect of PAI-1 antisense ODN on HG- (A) and TGF-β1- (B) increased fibronectin secretion in mesangial cells. Growth arrested and synchronized primary rat mesangial cells were stimulated with 30 mM high D-glucose (HG) (A) or 2 ng/ml of TGF-β1 (B) for 48 hours after PAI-1 antisense or mis-match sense ODN transfection. Fibronectin protein was detected by Western blot analysis as described in the Methods. Data are presented as means±SE of 4 experiments. *$p<0.05$ vs control without HG or TGF-β1, $^{\dagger}p<0.05$ vs HG or TGF-β1, C: control without HG or TGF-β1, AS: PAI-1 antisense ODN, MS: PAI-1 mis-match sense ODN.

in primary cultured rat mesangial cells. We successfully knocked down both basal and stimulated PAI-1 mRNA and protein expression in primary mesangial cells using 1 μM of phosphorothioate-modified antisense ODN. In human vascular endothelial and smooth muscle cells, 1 μM PAI-1 antisense ODN was effective to inhibit both basal and TGF-β1-induced PAI-1 expression [28].

PAI-1 down-regulation by antisense ODN was accompanied by increased both basal and suppressed plasmin and MMP-2 activity in our experimental condition. PAI-1 antisense ODN furthermore increased basal and TGF-β1-suppressed plasmin activity in human vascular endothelial and smooth muscle cells [28]. Therefore, these results support the notion that increased plasmin activity may play an important role in PAI-1 antisense ODN therapy limiting fibrosis. However, a recent study [27] demonstrated that treatment with PAI-1 decoy peptides reduced tubulointerstitial fibrosis in unilateral ureteral obstruction mice model without significant effect on plasmin activity but with increased PA activity along with hepatocyte growth factor (HGF) expression. The authors speculated HGF-dependent ECM degradation as an antifibrotic mechanism of PAI-1 decoy peptide.

ECM overproduction in diabetic nephropathy has been well documented in many studies, and decreased ECM degrading enzymes are also related with tissue fibrosis. In the present study, PAI-1 antisense ODN effectively suppressed basal and stimulated fibronectin protein secretion. As discussed above, the increment of plasmin and MMP-2 activity may have played a role in decreased fibronectin accumulation. On the other hand, PAI-1 can activate profibrotic protein synthesis via the uPA receptor [14,16]. We have also observed that recombinant PAI-1 stimulates TGF-β1 promoter activity and induces fibronectin and collagen I expression in mouse mesangial cells [16]. It is possible that PAI-1 antisense ODN-mediated fibronectin protein may be mediated by active suppression of ECM synthesis. The relative role of active suppression of ECM synthesis and increased ECM degradation in response to PAI-1 antisense ODN remains to be studied.

In summary, the present data demonstrated that PAI-1 antisense ODN effectively inhibited PAI-1 mRNA and protein expression, and subsequent changes in plasmin and MMP-2 activity in primary mesangial cells, and suggested that PAI-1 antisense ODN may be a potential therapeutic agent to inhibit renal PAI-1 upregulation and to prevent chronic kidney diseases including diabetic nephropathy. However, further study is necessary to investigate whether antifibrotic therapeutic potential of antisense ODN is better than those of small molecules or mutant PAI-1.

ACKNOWLEDGEMENTS

This work was supported by RP-Grant 2009 (2009-1949-1-1) from Ewha Womans University to Jehyun Park.

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