Original Article

Salutary Effect of Pigment Epithelium–Derived Factor in Diabetic Nephropathy

Evidence for Antifibrogenic Activities

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Diabetic nephropathy is a major complication of diabetes and a leading cause of end-stage renal diseases in the U.S. Pigment epithelium–derived factor (PEDF) is a potent angiogenic inhibitor that has been extensively studied in diabetic retinopathy. Recently, we reported that PEDF is expressed at high levels in normal kidneys and that PEDF levels are decreased in kidneys of streptozotocin (STZ)-induced diabetic rats. In the present study, we injected STZ-diabetic rats with an adenovirus expressing PEDF (Ad-PEDF) to evaluate its effects in diabetes. The results showed that increased expression of PEDF in the kidney in response to Ad-PEDF delivery significantly alleviated microalbuminuria in early stages of diabetes. Administration of Ad-PEDF was found to prevent the overexpression of two major fibrogenic factors, transforming growth factor-β (TGF-β)1 and connective tissue growth factor (CTGF), and to significantly reduce the production of an extracellular matrix (ECM) protein in the diabetic kidney. Moreover, PEDF upregulated metalloproteinase-2 expression in diabetic kidney, which is responsible for ECM degradation. In cultured human mesangial cells, PEDF significantly inhibited the overexpression of TGF-β1 and fibronectin induced by angiotensin II. PEDF also blocked the fibronectin production induced by TGF-β1 through inhibition of Smad3 activation. These findings suggest that PEDF functions as an endogenous anti–TGF-β and antifibrogenic factor in the kidney. A therapeutic potential of PEDF in diabetic nephropathy is supported by its downregulation in diabetes; its prevention of the overexpression of TGF-β, CTGF, and ECM proteins in diabetic kidney; and its amelioration of proteinuria in diabetic rats following Ad-PEDF injection. *Diabetes* 55:1678–1685, 2006

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Ad-GFP, adenovirus expressing green fluorescent protein; Ad-PEDF, adenovirus expressing pigment epithelium–derived factor; CTGF, connective tissue growth factor; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; HMC, human mesangial cell; MMP-2, matrix metalloproteinase-2; PEDF, pigment epithelium–derived factor; STZ, streptozotocin; TGF-β, transforming growth factor-β; UAE, urine albumin excretion.

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One major complication of diabetes is diabetic nephropathy, which occurs in 30–40% of the patients with type 1 diabetes and 10–20% of the patients with type 2 diabetes (1). In the U.S., diabetic nephropathy accounts for 30–40% of all end-stage renal disease cases (2), or ~28,000 new cases each year (2,3). Despite considerable advances in optimization of glycemic and blood pressure control, the incidence of diabetic nephropathy and subsequent end-stage renal disease has continued to climb (3). Thus, the search continues for a better understanding of the pathogenesis of diabetic nephropathy and for novel therapies.

Although the mechanisms are incompletely understood, mesangial extracellular matrix (ECM) accumulation and consequent renal fibrosis have been recognized as playing a major role in progressive renal failure in diabetic nephropathy (4,5). Several growth factors, such as transforming growth factor-β (TGF-β)1, connective tissue growth factor (CTGF), and vascular endothelial growth factor, are believed to be involved in the pathogenesis (5,6). In diabetic patients and animal models, mRNA and protein levels of TGF-β1 were found to be progressively increased in glomeruli, along with the increased productions of proteoglycans and other matrix components, in contrast to negative or barely detectable levels of TGF-β1 in glomeruli from normal kidneys (7). Blockade of the overexpression of TGF-β1 has been shown to prevent the pathological changes in diabetic nephropathy, such as thickening of the glomerular basement membrane and mesangial matrix expansion (8–10). Renal insufficiency could be prevented by the anti–TGF-β1 antibody in a mouse model of type 2 diabetes (10).

Pigment epithelial–derived factor (PEDF) is a glycoprotein belonging to the superfamily of serine proteinase inhibitors, first identified in cultured retinal pigment epithelial cells (11,12). It has been found to be an endogenous angiogenic inhibitor (13). Decreased PEDF levels have been associated with diabetic retinopathy (14,15). Our recent studies showed that PEDF is also expressed at high levels in normal rat kidneys, and renal PEDF levels are decreased in the streptozotocin (STZ)-induced diabetic rat model (16). Hyperglycemia is responsible for the decrease of PEDF in the diabetic kidneys (16). Moreover, PEDF prevents the overexpression of TGF-β1 and fibronectin induced by high-medium glucose in cultured human mesangial cells (HMCs) (16). These findings revealed the

implication of the decrease of PEDF in diabetic nephropathy. However, the protective effect of PEDF in diabetic kidney and its mechanism of action have not been demonstrated.

In the present study, we investigated the effects of PEDF delivered by an adenovirus vector in a rat model of STZ-induced diabetes. The results demonstrated that the PEDF treatment significantly ameliorated microalbuminuria, downregulated the expression of fibrogenic factors TGF-β1 and CTGF, and, subsequently, reduced mesangial matrix protein production in the kidney of diabetic rats.

RESEARCH DESIGN AND METHODS

Brown Norway (BN) rats were purchased from Charles River Laboratories (Wilmington, MA). Care, use, and treatment of all animals in this study were in strict agreement with the guidelines in the care and use of laboratory animals set forth by the University of Oklahoma.

Experimental diabetes was induced by an intraperitoneal injection of STZ (50 mg/kg in 10 mmol/l of citrate buffer; pH 4.5) into anesthetized BN rats (8 weeks of age) after an overnight fast. Age-matched control rats received an injection of citrate buffer alone. Blood glucose levels were measured 24 h after the STZ injection and monitored weekly thereafter. Only the animals with glucose levels >350 mg/dl were considered diabetic.

Preparation of adenovirus expressing human PEDF. A construct custom built for the expression of a full-length human PEDF and a green fluorescent protein (GFP) under a cytomegalovirus promoter in an adenoviral vector were obtained from a commercial source (InVivoGen, San Diego, CA). The construct sequence provided by the manufacturer was confirmed by DNA sequencing (OMRF, Oklahoma City, OK). The recombinant virus was amplified, purified, and titered in the 293A cell line following the procedure recommended by the manufacturer.

Intravenous delivery of adenovirus expressing human PEDF. One week after the STZ injection, diabetic rats were randomly assigned into three groups. Group 1 received no virus injection ($n = 5$); groups 2 and 3 received an intravenous injection of adenovirus expressing human PEDF (Ad-PEDF) ($n = 7$) and a control adenovirus expressing GFP under the CMV promoter (adenovirus expressing GFP [Ad-GFP]; Qbiogene, Montreal, Canada) ($n = 5$), respectively, at a dose of 4×10^{10} viral particles per rat.

Evaluation of rat microalbuminuria and measurement of creatinine. The 24-h urine was collected from each rat in individual metabolic cage and centrifuged at 2,000*g* for 5 min. To avoid the variations caused by increased urine volumes in diabetic rats, the total albumin in the 24 h was normalized by creatinine concentrations. Urine creatinine levels were measured using the QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA), following the manufacturer's protocol. Urine albumin was measured by enzyme-linked immunosorbent assay (ELISA) (Bethyl Laboratories, Montgomery, TX). Urine albumin excretion (UAE) was normalized by creatinine excretion and expressed as milligrams albumin per milligrams creatinine in 24-h urine.

Measurements of PEDF, TGF-β1, and fibronectin by ELISA. The protein levels of PEDF, TGF-β1, and fibronectin in kidney tissue homogenate, urine, or cultured cells were quantified using the commercial Quantikine PEDF ELISA kit (Chemicon, Temecula, CA), the TGF-β1 ELISA kit (R&D Systems, Minneapolis, MN), and the fibronectin ELISA kit (Assaypro, Winfield, MO), respectively, following the protocols from manufacturers. The ELISA results were normalized by total protein concentrations measured using the BioRad Protein Assay reagent.

Western blot analysis of CTGF. Western blot analysis was performed as described previously (16). Briefly, 50 μg of protein from each sample was blotted by an anti-CTGF antibody (R&D Systems). The same membranes were stripped and reblotted with an anti–β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Determination of the mRNA levels of matrix metalloproteinase-2 by real-time RT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The quality of the RNA samples was evaluated by electrophoresis through agarose gels with ethidium bromide staining. Primers specific for matrix metalloproteinase-2 (MMP-2) (MMP-2 forward, 5′-CTGAGCTCCCGGAAAAGATTG-3′; MMP-2 reverse, 5′-CCTGCGAAGAACACAGCCTT-3′) were used for real-time RT-PCR. PCR was performed using the GeneAmp RNA PCR kit and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The efficiency of real-time PCR was 99.1%. The average threshold cycle (C_T) of fluorescence units was used to analyze the mRNA levels. The mRNA levels of target genes were normalized by 18S ribosomal RNA levels. Quantification was calculated as mRNA levels (percent of control) $= 2^{\Delta(\Delta C_T)}$ with $\Delta C_T = C_{T\ \text{target gene}} - C_{T\ 18S\ RNA}$ and $\Delta(\Delta C_T) = \Delta C_{T\ \text{normal sample}} - \Delta C_{T\ \text{STZ-diabetic sample}}$.

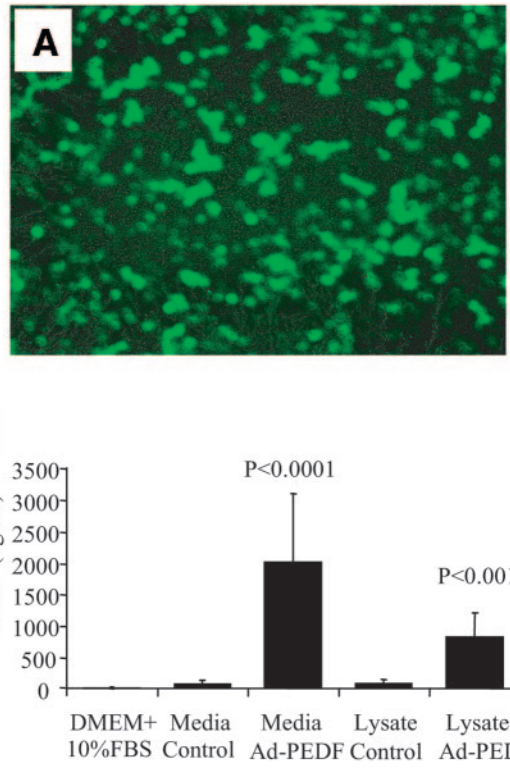

FIG. 1. PEDF expression mediated by adenovirus vector. The 293 cell line was infected with Ad-PEDF. The infection efficiency was monitored using GFP as a marker under a fluorescent microscope, 3 days after the infection (*A*). PEDF protein levels in the conditioned media and in the cell lysates were measured by ELISA (mean ± SD, $n = 3$) (*B*). DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

Smad nuclear translocation assay. Primary HMC were cultured on 4-chamber slides (Nalge Nunc, Naperville, IL) to reach 80% confluence. After exposure to 1.25 ng/ml TGF-β1 with or without 160 nmol/l PEDF for 1 h, the cells were immediately fixed with 4% paraformaldehyde. The cells were incubated with an anti-Smad2/3 antibody (1:200; Upstate, Lake Placid, NY) for 2 h and then incubated with a Cy3-conjugated donkey anti-rabbit IgG antibody for 1 h. The slide was examined and analyzed under a fluorescent microscope (Olympus, Humburg, Germany).

Statistical analysis. Data were calculated and expressed as group means ± SD. Statistical analyses were performed using Student's t test, ANOVA, and Bonferroni's multiple comparison test. Statistical difference was considered significant at a P value of <0.05.

RESULTS

High levels of PEDF expression mediated by Ad-PEDF in cultured cells. To confirm the expression of PEDF mediated by Ad-PEDF, 293A cells were infected with Ad-PEDF. Three days following the viral infection, the cultured cells showed high levels of GFP, a marker of the Ad-PEDF vector. PEDF protein levels were measured in the culture media and cell lysates of the cells infected by Ad-PEDF using a commercial ELISA. The results showed significantly higher PEDF levels in the conditioned media from the cells infected by Ad-PEDF ($P < 0.0001$, $n = 3$). The lysates from the cells infected with Ad-PEDF also showed significantly increased PEDF levels over the control ($P < 0.001$, $n = 3$) (Fig. 1).

Increased PEDF levels in the kidney and urine in diabetic rats after the Ad-PEDF delivery. Either Ad-PEDF or the control virus Ad-GFP was injected into the tail vein of STZ-induced diabetic rats 1 week following the

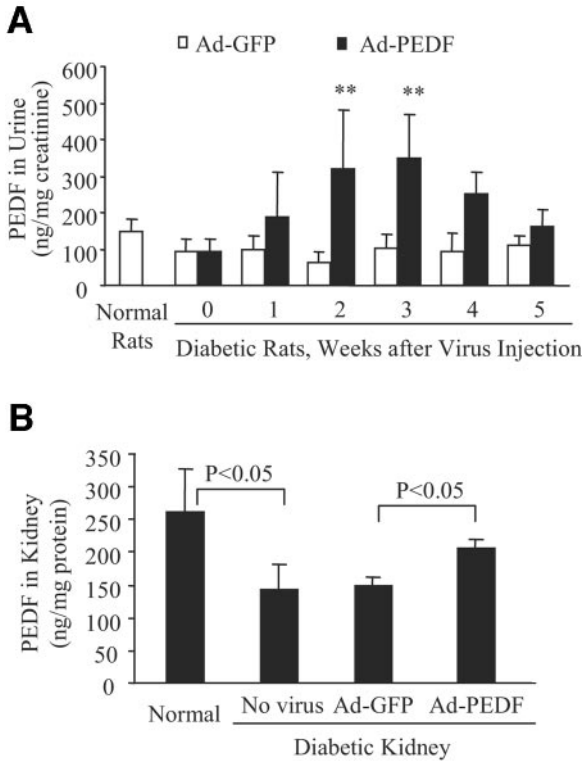

FIG. 2. Urine PEDF excretion and PEDF levels in the kidney of diabetic rats after the Ad-PEDF treatment. *A*: The 24-h urine was collected weekly from the diabetic rats before and after the injection of Ad-PEDF or Ad-GFP. PEDF levels in the urine were measured by ELISA and normalized by urine creatinine excretion and expressed as nanograms per milligram creatinine (mean ± SD, n = 5–7). Values statistically different from the Ad-GFP–treated controls are indicated by **$P < 0.001$. *B*: PEDF levels were measured in the kidney of the diabetic rats 3 weeks after the virus delivery and in age-matched nondiabetic normal rats using ELISA and normalized by total protein concentrations (means ± SD, n = 4). The results showed that renal PEDF levels were significantly decreased in diabetic kidneys, when compared with the nondiabetic controls ($P < 0.05$, n = 4). There was no difference in the PEDF levels between Ad-GFP–treated and untreated diabetic rats. Significant elevation of PEDF levels was observed in the Ad-PEDF–treated diabetic rats, when compared with Ad-GFP–treated or untreated diabetic rats ($P < 0.05$, n = 4).

onset of diabetes. The 24-h urine was collected from each rat individually at 0, 1, 2, 3, 4, and 5 weeks following the injection of Ad-PEDF. Total amounts of PEDF protein in 24-h urine was measured by ELISA and normalized by urine creatinine. The PEDF levels in the urine were significantly increased in diabetic rats injected with Ad-PEDF versus those injected with Ad-GFP, at 2 and 3 weeks after the virus injection (Bonferroni's post hoc test, $P < 0.001$, t = 4.700 and 4.273, respectively; Fig. 2*A*). PEDF levels in the urine declined to control levels after 4 weeks ($P > 0.05$; Fig. 2*A*), consistent with the course of transient gene expression from adenoviral vectors.

Three weeks following the virus injection, rats were killed, and PEDF levels in the kidney were measured using ELISA. Renal PEDF levels in untreated diabetic rats were significantly reduced versus age-matched nondiabetic normal rats (ANOVA, P = 0.01, F = 6.774; Fig. 2*B*), corroborating our previous report (16). Ad-PEDF injection restored PEDF levels in the kidney of diabetic rats to a level approaching that of normal controls (Bonferroni's post hoc test, $P > 0.05$, t = 1.646 for diabetic rats treated with Ad-PEDF versus normal controls; Fig. 2*B*). In contrast, injection of the same titer of the control virus, Ad-GFP, did not normalize the decrease of PEDF levels in diabetic rats (Bonferroni's post hoc test, $P < 0.05$, t = 3.556 for diabetic rats treated with Ad-GFP versus normal controls; Fig. 2*B*), suggesting that the restoration of PEDF levels by Ad-PEDF is not a nonspecific effect from the adenovirus infection.

PEDF gene delivery did not change blood glucose level, body weight, or urine volume in diabetic rats. Compared with age-matched normal rats, the STZ-induced diabetic rats had significantly elevated blood glucose concentrations (442.00 ± 57.67 mg/dl) 48 h after STZ injection and thereafter. At 1 week after diabetes onset, no significant difference in body weight was observed between the diabetic rats (128.55 ± 5.37 g) and age-matched control rats (130 ± 9.21 g). At 10 weeks after diabetic onset, the body weights of diabetic rats (138.7 ± 27.4 g) were significantly lower than that of age-matched control rats (192.8 ± 3.7 g, $P < 0.01$). Ad-PEDF injection did not alter the hyperglycemia or the body weight when compared with diabetic rats treated with Ad-GFP (ANOVA, $P > 0.05$, F = 0.9492 and 1.095, respectively; Table 1). As expected, the diabetic rats had polyuria versus the normal controls. At 1 week after diabetes onset, the urine volume in diabetic rats (46.7 ± 12.9 ml) was significantly higher than that in age-matched normal controls (5.2 ± 0.8 ml, $P < 0.001$). There was, however, no difference in 24-h urine volume between diabetic rats treated with Ad-PEDF and those treated with Ad-GFP ($P > 0.1$, n = 4–7; Table 2).

Decreased UAE by Ad-PEDF in diabetic rats. Microalbuminuria is an early clinical manifestation of diabetic nephropathy (17). In the present study, albumin in 24-h urine was measured using ELISA specific for rat albumin and normalized by the amount of urine creatinine. The results demonstrated that diabetic rats developed a significant microalbuminuria with onset as early as 1 week after the STZ injection (Bonferroni's post hoc test, $P < 0.05$, t = 2.535; Fig. 3). The 24-h UAE continued to increase

TABLE 1
Clinical characteristics of diabetic rats after PEDF virus treatment

Time after virus injection (weeks)	Blood glucose (mg/dl)		Body weight (g)	
	Ad-GFP	Ad-PEDF	Ad-GFP	Ad-PEDF
0	427.0 ± 40.4	435.6 ± 79.9	128.6 ± 2.1	131.0 ± 8.1
1	431.0 ± 28.9	461.5 ± 88.7	127.3 ± 3.6	133.7 ± 24.6
2	482.2 ± 81.2	437.7 ± 75.0	136.6 ± 9.2	137.2 ± 18.2
3	419.6 ± 39.9	449.0 ± 80.9	137.2 ± 8.7	146.7 ± 18.5
4	412.0 ± 39.9	489.4 ±136.7	139.0 ± 2.6	134.8 ± 28.1
7	490.7 ± 33.3	539.5 ± 78.5	141.3 ± 13.9	137.7 ± 36.8
9	538.3 ± 68.9	472.5 ± 47.4	139.3 ± 15.5	138.0 ± 40.4

Data are means ± SD, n = 4–7.

TABLE 2
Twenty-four–hour urine volume in diabetic rats after PEDF virus treatment

Time after virus injection (weeks)	24-h urine volume (ml)	
	Ad-GFP	Ad-PEDF
0	46.7 ± 12.9	40.1 ± 13.4
1	20.6 ± 5.9	45.7 ± 14.3
2	23.0 ± 4.5	39.0 ± 8.2
3	45.2 ± 5.6	44.8 ± 10.9
4	16.7 ± 1.5	47.6 ± 19.2
5	44.0 ± 3.6	45.8 ± 16.6
7	80.0 ± 14.8	45.7 ± 29.5
9	56.3 ± 11.6	52.0 ± 40.1

Data are means ± SD, $n = 4$–7.

with time through the 5 weeks analyzed in rats treated with the control virus (ANOVA, $P < 0.0001$, $F = 20.09$; Fig. 3). The diabetic rats injected with Ad-PEDF showed significantly reduced UAE at 2, 3, and 4 weeks after the injection of the Ad-PEDF versus diabetic rats injected with Ad-GFP (ANOVA, $P < 0.0001$, $F = 15.3$; Bonferroni's post hoc test, $P < 0.01$, $t = 4.525$, 4.336, and 4.740; $n = 4$–7; Fig. 3).

Inhibition of mesangial matrix protein accumulation in diabetic kidney by PEDF gene delivery. It is well known that the accumulation of ECM proteins in the kidney and subsequent mesangial expansion are typically and consistently present in diabetic nephropathy, parallel with microalbuminuria (4,5). Four weeks after the STZ injection, fibronectin levels in the kidney were significantly increased in the diabetic rats treated with the control virus Ad-GFP when compared with nondiabetic rats (ANOVA, $P < 0.0001$, $F = 54.99$; Fig. 4*A*). At the same time, the Ad-PEDF injection decreased kidney fibronectin levels in the diabetic kidney to the normal range (Bonferroni's post hoc test, $P > 0.05$, $t = 1.192$ for diabetic rats treated with Ad-PEDF versus normal controls; Fig. 4*A*).

It has been shown that both a decreased ECM protein degradation and an increased ECM protein expression contribute to the accumulation of mesangial ECM in the diabetic kidney (18). As MMP-2 is a major enzyme responsible for the degradation of ECM (19), we determined whether the PEDF gene delivery affects the expression of MMP-2 in the kidney. The results showed that in the diabetic kidney, MMP-2 expression was decreased to 60% of that in the kidney of normal controls ($P < 0.05$, $n = 4$; Fig. 4*B*). The PEDF gene delivery significantly increased MMP-2 mRNA levels in the diabetic kidney when compared with that in the Ad-GFP control group ($P < 0.05$, $n = 4$; Fig. 4*B*).

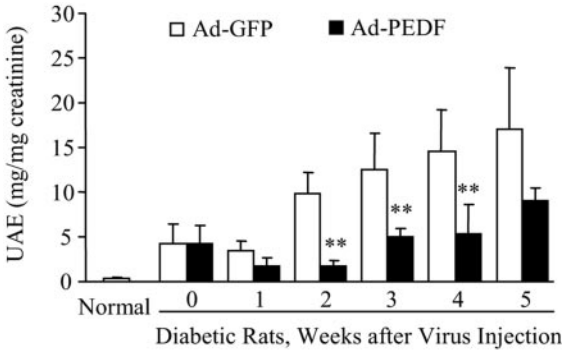

FIG. 3. Decreased UAE after the Ad-PEDF treatment in diabetic rats. The 24-h urine was collected weekly from the diabetic rats before and after the injection of Ad-PEDF or Ad-GFP. The albumin in the urine was measured by ELISA and normalized by creatinine levels in the urine and expressed as milligrams per milligram creatinine (mean ± SD, $n = 5$). Values statistically different from the Ad-GFP–treated controls are indicated by $^{**}P < 0.01$.

Decreased urine TGF-β1 excretion and kidney TGF-β1 levels in diabetic rats by PEDF gene delivery. TGF-β1 is a major growth factor mediating the remodeling of ECM and mesangial expansion in diabetic nephropathy. In this study, we investigated the effect of PEDF on urine TGF-β1 excretion and TGF-β1 protein levels in diabetic kidneys. The results showed that the TGF-β1 excretion in the urine was significantly attenuated at 2 and 3 weeks after the Ad-PEDF injection (Fig. 5*A*).

TGF-β1 levels in the kidney, as measured by ELISA, were significantly elevated in the diabetic rats treated with the control virus, Ad-GFP, compared with that in the nondiabetic normal rats. Such overexpression of TGF-β1 was effectively prevented by the PEDF gene delivery ($P < 0.05$, $n = 4$; Fig. 5*B*).

Decreased CTGF expression in the diabetic kidney by PEDF gene delivery. CTGF is an important mediator of fibrosis by promoting the glomerular matrix accumulation in diabetic nephropathy (20). In the present study, the expression of CTGF was measured at the protein level. As shown by Western blot analysis, CTGF expression was significantly increased in diabetic kidney at the protein level ($P < 0.001$; Fig. 6). Ad-PEDF treatment significantly downregulated CTGF expression when compared with the diabetic rats treated with the control virus ($P < 0.001$; Fig. 6). These findings suggest that the reduction of the ECM protein accumulation by the PEDF gene delivery is mediated at least in part by inhibition of CTGF expression in the diabetic kidney.

PEDF inhibited TGF-β1 and fibronectin secretion from HMCs induced by angiotension II. To determine the direct effect of PEDF on fibrosis, we used primary HMCs treated with angiotensin II, which is known to play a pathogenic role in diabetic nephropathy and induce TGF-β1 expression. As measured by ELISA, PEDF, at concentrations of 40 and 160 nmol/l, significantly abrogated the stimulation of TGF-β1 expression by angiotensin II in cultured HMCs (Fig. 7). Similarly, PEDF also significantly prevented the induction of fibronectin secretion by angiotensin II in HMCs (Fig. 7), suggesting a direct effect of PEDF on fibrosis in kidney cells.

PEDF abolished TGF-β1–induced fibronectin secretion via the blockade of Smad2/3 nuclear translocation in HMCs. To gain insights into the antifibrogenic activity of PEDF, we have measured the direct effect of PEDF on fibronectin overexpression stimulated by TGF-β1 in cultured HMCs. As shown in Fig. 8, TGF-β1 treatment induced significant fibronectin overexpression in HMCs. At concentrations of 40–640 nmol/l, PEDF prevented the stimulation of fibronectin expression by TGF-β1 in a concentration-dependent manner.

To elucidate the mechanism responsible for the antifibrogenic activity of PEDF, we measured the activation of Smad3 pathway, which is known to mediate the effect of TGF-β1 on fibrosis (21,22). As Smad3 nuclear translocation is a crucial step in the activation of the pathway, we measured the PEDF effect on Smad3 nuclear translocation induced by TGF-β1. As shown by immunocytochemistry using an antibody specific for Smad2/3, Smad2/3 signal was diffusely distributed in the cytoplasm of the control

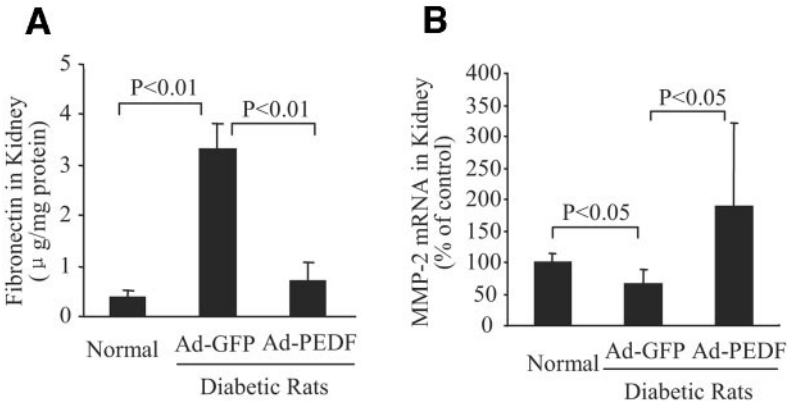

FIG. 4. Decreased fibronectin levels and increased MMP-2 expression in the diabetic kidney after the PEDF gene delivery. *A*: Three weeks after the virus injection, fibronectin levels in the kidney were measured by ELISA (means ± SD, $n = 4$). *B*: MMP-2 mRNA levels were determined by quantitative real-time RT-PCR and expressed as percent of that in nondiabetic control (means ± SD, $n = 4$).

HMCs. Upon treatment with TGF-β1, Smad2/3 levels were apparently increased and translocated into the nucleus. PEDF effectively blocked the translocation of Smad2/3 into the nucleus in HMCs treated with TGF-β1, suggesting that the antifibrogenic action of PEDF may be through interference with the Smad signaling pathway of TGF-β1.

DISCUSSION

PEDF is a major angiogenic inhibitor in the eye (13). The pathogenic role of the decreased retinal PEDF levels in vascular leakage and retinal neovascularization in models of diabetic retinopathy has been established (23,24). We recently reported that PEDF is highly expressed in the normal kidney but reduced in kidneys of diabetic rats, suggesting a potential implication of decreased PEDF levels in diabetic nephropathy (16). However, the protective effect of PEDF against abnormalities of diabetic nephropathy has not been demonstrated in vivo. In the present study, we provide the first in vivo evidence that a PEDF gene delivery ameliorates proteinuria in rats with STZ-induced diabetes. Toward potential mechanism for the PEDF effect, we found that PEDF inhibits the expression of pathogenic factors TGF-β1 and CTGF and suppresses ECM protein production in diabetic kidney, suggesting an antifibrogenic activity. Therefore, these observations revealed a new antifibrogenic activity of PEDF, which may be responsible for its salutary effect in diabetic nephropathy.

Microalbuminuria is a characteristic feature of early stages of diabetic nephropathy. It has been shown to be a good predictive feature for the progression to overt dia-

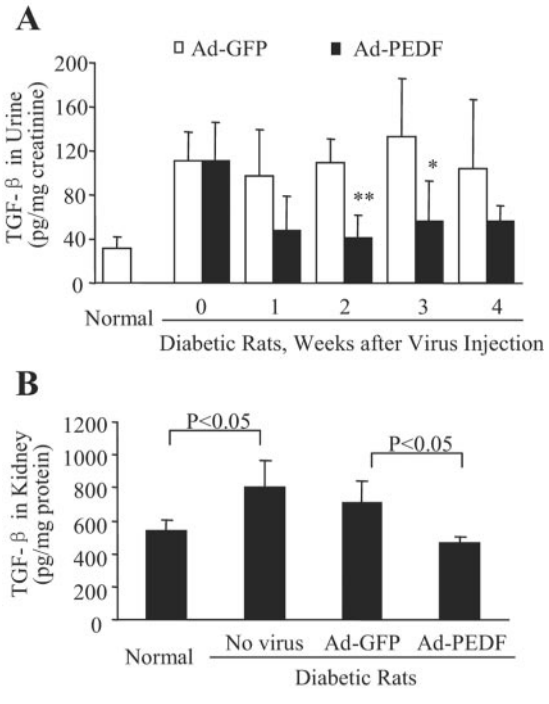

FIG. 5. Decreased urine TGF-β1 excretion and TGF-β1 levels in the kidney after the Ad-PEDF treatment. *A*: Total TGF-β1 levels in 24-h urine from diabetic rats were measured by ELISA at 1, 2, 3, and 4 weeks after the Ad-PEDF injection. The results were normalized by urine creatinine excretion and expressed as picograms per milligram creatinine (means ± SD, $n = 5$). Values statistically different from the Ad-GFP–treated controls are indicated by *$P < 0.05$ and **$P < 0.01$. *B*: TGF-β1 levels in the kidney were measured by ELISA in the diabetic rats 3 weeks after the virus delivery (means ± SD, $n = 4$).

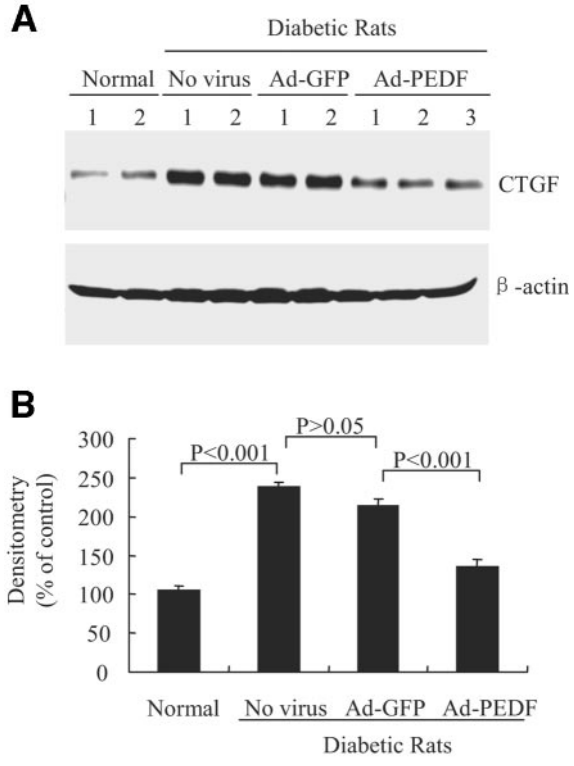

FIG. 6. Decreased CTGF expression in the kidney of diabetic rats after the Ad-PEDF treatment. At 3 weeks after the virus injection, the expression of CTGF in the kidney was determined at the protein level by Western blot analysis using the same amount of total proteins from the kidney (*A*). The results were normalized by β-actin levels and semiquantified by densitometry (*B*). Values in *B* are means ± SD ($n = 4$).

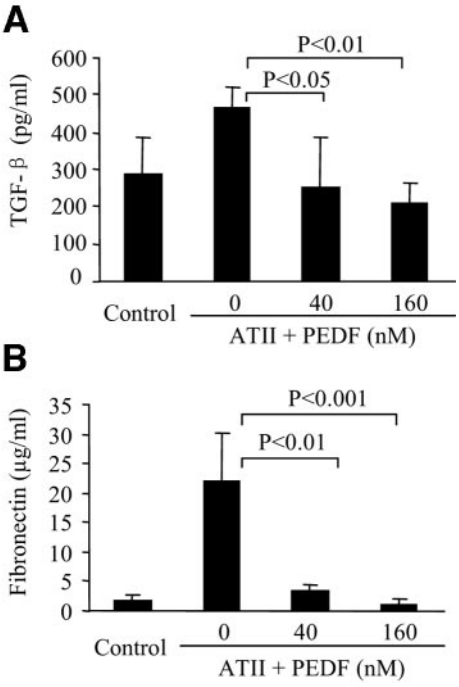

FIG. 7. PEDF inhibited TGF-β1 and fibronectin secretion from HMCs treated with angiotension II (ATII). HMCs were incubated with 50 nmol/l of angiotension II in the absence or presence of PEDF (40–160 nmol/l) for 24 h. TGF-β1 (*A*) and fibronectin (*B*) secreted into the culture medium were measured by ELISA, normalized by total protein concentrations, and expressed as picograms or micrograms per milligram total protein (means ± SD, *n* = 3).

betic nephropathy (3,25,26). Recent studies indicate that microalbuminuria is an independent risk factor for cardiovascular complications and mortality in type 2 diabetic patients (27,28). Therefore, the prevention and treatment of microalbuminuria are important therapeutic targets in halting the progression of microvascular and macrovascular diabetes complications. The present study demonstrated that PEDF gene delivery significantly decreased microalbuminuria in diabetic rats. Although this PEDF effect lasted only for 3 weeks due to the inherently transient expression mediated by the adenoviral vector, the result suggests that the PEDF treatment may be promising in preventing progressive renal injury and consequently reducing the risk for overt nephropathy.

Mesangial matrix expansion is the most prominent pathological feature of diabetic nephropathy, which is characterized by the accumulation of ECM proteins such as collagen IV and fibronectin in the glomeruli (29–31). In diabetes, both the accelerated production and the decreased degradation of ECM proteins are known to contribute to the accumulation of mesangial matrix (29,32). Accumulating evidence suggests that MMP-2 is an important protease degrading ECM (18,33). In the kidney, MMP-2 is highly expressed in glomerular mesangial cells and moderately in epithelial cells (19). In high-glucose medium conditions, the expression and activity of MMP-2 were decreased in glomerular mesangial cells, which could lead to reduced ECM degradation and subsequent mesangium accumulation (18,33). Our study demonstrated that the upregulation of tissue fibronectin in the diabetic kidney is effectively reversed after the PEDF treatment. These results are consistent with but extend our previous findings showing that PEDF inhibits high glucose–induced fibronectin secretion in cultured HMCs (16), suggesting that PEDF is a potent inhibitor of ECM production by mesangial cells. Additionally, we observed that MMP-2 expression was significantly downregulated in the diabetic kidney but restored to nondiabetic control levels by the PEDF gene delivery. These findings suggest that PEDF suppresses glomerular mesangial matrix accumulation possibly by two mechanisms, inhibiting ECM production and enhancing ECM degradation.

TGF-β refers to three isoforms of secreted homodimeric proteins encoded by different genes (34). In the process of tissue development and wound healing, TGF-β plays a crucial role in controlling the ECM deposition and remodeling (22). On one hand, TGF-β stimulates the synthesis of

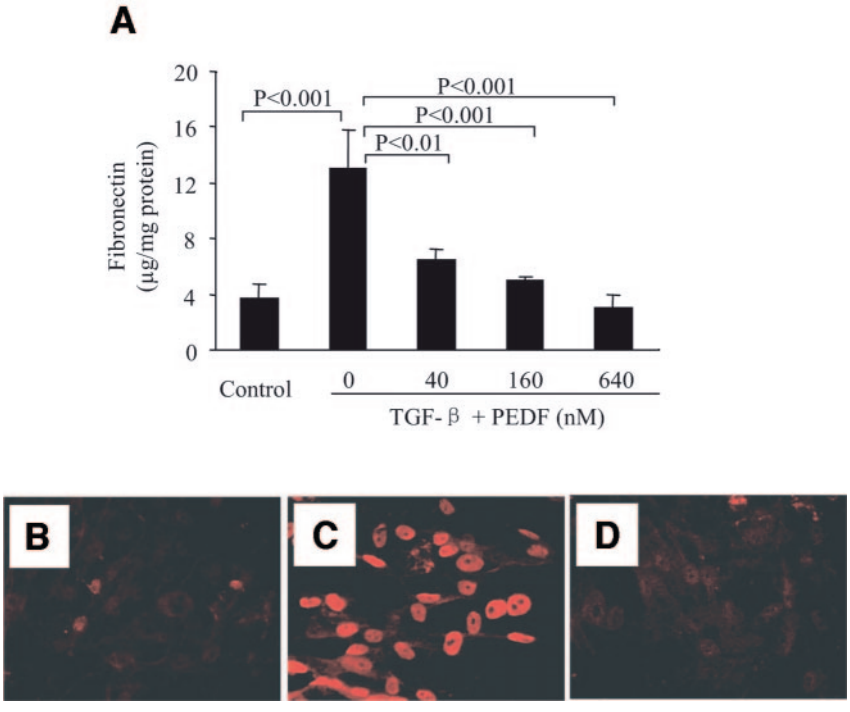

FIG. 8. PEDF inhibited fibronectin secretion and Smad2/3 nuclear translocation induced by TGF-β1 in HMCs. *A*: HMCs were incubated with 5 ng/ml TGF-β1 in the absence or presence of various concentrations of PEDF (40–640 nmol/l) for 24 h. Fibronectin secreted into the medium was measured by ELISA, normalized by total protein concentrations, and expressed as micrograms per milligram total protein (means ± SD, *n* = 3). *B*: Effects of PEDF on Smad2/3 translocation in HMCs. HMCs were plated onto glass coverslips. After an exposure to a serum-free medium for 24 h, the cells were treated with 1.25 ng/ml TGF-β1 in the absence or presence of 160 nmol/l PEDF for 1 h. The cells were fixed and stained with an anti-Smad2/3 antibody and visualized under a fluorescent microscope (400×). *B*: Control cells. *C*: Cells treated with TGF-β1 alone. *D*: Cells treated with TGF-β1 and PEDF. Significant increases of Smad2/3 expression and nuclear translocation were observed in the cells exposed to TGF-β1 (*C*), when compared with that in the control cells (*B*). PEDF effectively blocked the TGF-β1–induced upregulation and translocation of Smad2/3 (*D*).

major components of ECM proteins, such as collagen and fibronectin (29,35). On the other hand, TGF-β inhibits ECM degradation by decreasing protease secretion and activating protease inhibitors (36) and tissue inhibitor of metalloproteinase-1 (37). In diabetic kidneys, the overexpression of TGF-β is believed to be the major mediator responsible for the early pathological changes of diabetic nephropathy, including the glomerular basement membrane thickening and mesangial matrix expansion (9,20,34,38–40). Blockade of TGF-β expression or function has been shown to ameliorate pathological damage or to prevent functional renal insufficiency in diabetic nephropathy (9,10,29,41). The present study demonstrated that the PEDF gene delivery effectively decreased the urine TGF-β1 excretion and TGF-β1 expression in the kidney, providing the first in vivo evidence suggesting that PEDF functions as an endogenous inhibitor of TGF-β expression. These data suggest that the reduction of ECM accumulation induced by PEDF may be mediated, at least in part, by the downregulation of TGF-β1.

The Smad pathway is known to mediate the functions of TGF-β on renal fibrogenesis and subsequent ECM accumulation in diabetic nephropathy (13). The present study demonstrated that TGF-β induces Smad2/3 nuclear translocation, a key step in the activation of the Smad pathway. Moreover, PEDF blocks the Smad2/3 nuclear translocation induced by TGF-β in HMCs. This result suggests that PEDF not only downregulates TGF-β expression but also blocks its function, likely through the Smad pathway.

A number of studies have indicated that CTGF, a 349–amino acid cysteine-rich peptide, is an important downstream mediator of the fibrogenic effect of TGF-β in regulating matrix metabolism (42–44). All of the causal factors of diabetic glomerulosclerosis, including TGF-β and high glucose concentration, significantly induced CTGF expression in glomerular mesangial cells (45). In the early stage of diabetic nephropathy in *db/db* mice, the CTGF expression in the glomeruli is significantly increased (45). In a recent study in type 1 diabetic patients with nephropathy, the urine CTGF excretion was found to correlate with the severity of diabetic nephropathy (46). These findings support the notion that CTGF is an important mediator of diabetic kidney disease. In the present study, we demonstrated the upregulation of CTGF expression in the kidneys of STZ-induced diabetic rats, consistent with the previous observations from *db/db* mice and diabetic patients (45,46). Furthermore, we noted that the PEDF treatment essentially abrogated the overexpression of CTGF in diabetic kidneys, concomitant with the normalization of TGF-β1 and fibronectin levels to the nondiabetic range. These findings suggest that PEDF may suppress mesangial matrix accumulation through the blockade of hyperglycemia-induced TGF-β1 and CTGF expression.

In summary, our data provide the first evidence suggesting that PEDF blocks fibrogenesis in diabetic kidneys, via inhibition of TGF-β and CTGF expression and function. The antifibrogenic activity of PEDF may be responsible, at least in part, for its salutary effects in diabetic kidney complication in a type 1 diabetic rat model. As previous studies have shown that anti–TGF-β therapies can ameliorate the functional and structural abnormalities of diabetic nephropathy, we propose that PEDF, by virtue of its anti–TGF-β and antifibrogenic properties, may have a therapeutic potential in diabetic nephropathy.

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