Anti-inflammatory effects of pigment epithelium-derived factor in diabetic nephropathy

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DIABETIC NEPHROPATHY (DN) is one of the most common and severe microvascular complications of diabetes. Early characteristics of diabetic nephropathy are albuminuria, glomerular hypertrophy, and thickening of the glomerular basement membrane (GBM) (26). The progressive changes in the diabetic kidney, i.e., mesangial matrix expansion and eventual glomerular sclerosis, lead to a relentless decline in glomerular filtration rate (GFR) until end-stage renal disease (ESRD) (26). Although many therapeutic interventions have been shown to delay the development or retard the progression of diabetic nephropathy, currently no intervention has been able to halt or reverse its progression. Therefore, better therapeutic modalities are urgently needed.

In recent years, accumulating evidence demonstrates that the pathogenesis of diabetic nephropathy is associated with low-grade inflammation. Increased levels of some proinflammatory factors such as intercellular adhesion molecule-1 (ICAM-1) and monococyte chemotactant protein-1 (MCP-1) have been found in both type 1 and type 2 diabetic patients with nephropathy (32, 36, 40). In human biopsy samples, it has been shown that increased macrophage infiltration is involved in the pathogenesis of diabetic nephropathy (12). The causative role of inflammation in diabetic nephropathy has been confirmed using the ICAM-1 gene knockout mouse. Knockout of the ICAM-1 gene significantly reduced macrophage infiltration into the kidney after induction of diabetes. Moreover, ICAM-1 deficiency also ameliorated renal structural and functional abnormalities in diabetic mice (32). These results support the notion that inflammation plays an important role in the development and progression of diabetic nephropathy.

Pigment epithelium-derived factor (PEDF) is a neurotrophic and a potent angiogenic inhibitor (8). Decreased PEDF levels have been shown to be pathogenic in diabetic retinopathy (DR) (13, 39). Systemic or local delivery of recombinant PEDF protein or viral vector-mediated PEDF gene therapy successfully inhibited retinal neovascularization and reduced retinal vascular permeability in diabetic animals. In addition, PEDF has also been recognized as an important endogenous anti-inflammatory factor (47). Loss or decreased levels of PEDF in the retina may contribute to the pathogenesis of DR by augmentation of retinal inflammation (47).

Our recent studies have demonstrated that decreased PEDF levels in the kidney are implicated in diabetic nephropathy (45). Moreover, systemic administration of an adenovirus expressing the PEDF gene has shown a salutary role on prevention of nephropathy, i.e., drastically reducing albuminuria and ameliorating glomerular hypertrophy in a rat model of type 1 diabetes (46). Moreover, PEDF inhibited the expression of fibronectin in the diabetic kidney. However, the mechanisms for the protective effect of PEDF in the diabetic kidney are undefined. Based on the causative role of inflammation in diabetic nephropathy and the anti-inflammatory activity of PEDF, it is logical to hypothesize that PEDF protects the renal structure and function from diabetic injury via its anti-inflammatory activity. We have tested this hypothesis in the present study.

RESEARCH DESIGN AND METHODS

Animals. Brown Norway rats were purchased from Charles River Laboratories (Wilmington, MA). The care, use, and treatment of all animals in this study were in strict agreement with the guidelines in the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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the care and use of laboratory animals set forth by the University of Oklahoma.

**Induction of experimental diabetes.** Diabetes was induced by an intraperitoneal injection of STZ (50 mg/kg in 10 mM of citrate buffer, pH 4.5) into Brown Norway rats (8 wk of age) after an overnight fast. Age-matched control rats received an injection of citrate buffer alone. Blood glucose levels were measured 2 days after the STZ injection and monitored weekly thereafter. Only the animals with glucose levels higher than 300 mg/dl were considered diabetic. The 24-h urine was collected from each rat in an individual metabolic cage and stored at −80°C after centrifugation at 2,000 g for 5 min.

**Cell culture.** Primary human glomerular mesangial cells (HMC) were purchased from Cambrex Bio Science (Walkersville, MD). The HMC were cultured in the mesangial cell basal medium (Cambrex) with 0.1% GA-1000 (Cambrex) and 5% fetal bovine serum at 37°C in a humidified 5% CO₂ atmosphere. Cells from passages 8–10 were used in the experiments. For immunocytochemical study, the cells were grown onto the eight-well chambered slides (Nalgé Nunc International, Naperville, IL). After reaching 80% confluence, cells were exposed to medium with 0.5% serum for 12 h and treated with desired agents.

**Preparation and intravenous delivery of an adenovirus expressing human PEDF.** A recombinant adenovirus expressing human PEDF (Ad-PEDF) was constructed by cloning a full-length human PEDF cDNA under the control of the CMV promoter (46). The construct sequence was confirmed by DNA sequencing (OMRF, Oklahoma City, OK). A control virus containing a green fluorescent protein (Ad-GFP, Qbiogene, Montreal, QC, Canada) under the same promoter which was obtained from a commercial source (InVivoGen, San Diego, CA). The recombinant virus was amplified, purified, and titred as described previously (46). One week after the STZ injection, diabetic rats were randomly assigned into three groups. Group 1 received no virus injection (n = 5), and groups 2 and 3 received an intravenous injection of Ad-PEDF (n = 7) and Ad-GFP (n = 5), respectively, at a titer of 4×10⁹ viral particles/rat.

**Measurements of ICAM-1, MCP-1, TNF-α, and VEGF by ELISA.** The protein levels of ICAM-1, MCP-1, TNF-α, and VEGF in kidney tissue homogenate, in conditioned cell culture media were measured using commercial ELISA kits from R&D Systems (Minneapolis, MN). The samples and presented as 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**

**General clinical data and effects of PEDF gene delivery on albuminuria and kidney matrix protein deposition in diabetic rats.** As reported previously, STZ-diabetic rats had significantly elevated blood glucose concentrations (300–500 mg/dl), decreased body weight, apparent polyuria, and proteinuria (46). An intravenous injection of Ad-PEDF did not alter the hyperglycaemia, body weight, or polyuria at 1, 2, and 3 wk following the injection compared with diabetic rats injected with Ad-GFP. At 2 and 3 wk after the Ad-PEDF injection,
UAE was significantly decreased compared with the diabetic rats injected with Ad-GFP. Renal levels of fibronectin, a major component of ECM proteins as a marker of matrix expansion, were significantly decreased in the diabetic rats treated with Ad-PEDF (46). Ad-PEDF delivery inhibits VEGF overexpression in the kidney of diabetic rats. VEGF is a potent angiogenic factor and a vascular permeability factor (VPF) (1, 9). It is also recognized as a proinflammatory factor inducing ICAM-1 expression in vitro and in vivo (25, 30). VEGF expression has been shown to be upregulated at early stages of diabetic nephropathy in both diabetic patients and animal models (10, 22, 47). To determine whether the PEDF-induced reduction of albuminuria is mediated by downregulation of VEGF expression in diabetic kidneys, we have compared renal VEGF levels in the kidneys of Ad-PEDF-treated diabetic rats with those treated with Ad-GFP. The results showed that the renal VEGF levels were significantly elevated in diabetic rats, consistent with the increase in renal inflammatory factors ICAM-1, MCP-1, and TNF-α. The Ad-PEDF treatment significantly reduced the protein levels of VEGF in the kidney of diabetic rats compared with that in Ad-GFP-treated rats ($P < 0.05$, $n = 5$, Fig. 2B). The overexpression of the VEGF mRNA was also significantly blocked by Ad-PEDF (Fig. 2A).

**PEDF decreases high glucose-induced MCP-1 secretion in HMC.** MCP-1 is one of the most important proinflammatory chemokines implicated in the pathogenesis of diabetic nephropathy (2, 19, 44). High glucose is known to upregulate MCP-1 expression via NF-κB activation in cultured renal mesangial cells (15). In the present study, we examined the effects of PEDF on MCP-1 secretion induced by a high-glucose medium. After incubation with a high-glucose medium for 48 h, the MCP-1 secretion was increased by 1.7-fold over that of the low-glucose control (Fig. 3A). PEDF at 10 nM significantly blocked the increase in MCP-1 induced by high glucose. The expression of MCP-1 at the mRNA level was also significantly inhibited by PEDF (Fig. 3B).

**PEDF inhibits NF-κB nuclear translocation in HMC cultured in a high-glucose medium.** As NF-κB is the major transcription factor regulating a variety of inflammatory factors, including MCP-1 and ICAM-1 (6, 20, 29, 36, 42), we further investigated whether the inhibitory effects of PEDF on MCP-1 and ICAM-1 expression are through the inhibition of NF-κB activation. HMC were exposed to 30 mM glucose in the culture medium or the same concentration of mannitol as an osmolarity control for 48 h. PEDF was added into the medium at high glucose (Fig. 4A, -), induced nuclear translocation in HMC cultured in a high-glucose medium. As NF-κB is the major transcription factor regulating a variety of inflammatory factors, including MCP-1 and ICAM-1 (6, 20, 29, 36, 42), we further investigated whether the inhibitory effects of PEDF on MCP-1 and ICAM-1 expression are through the inhibition of NF-κB activation. HMC were exposed to 30 mM glucose in the culture medium or the same concentration of mannitol as an osmolarity control for 48 h. PEDF was added into the medium at high glucose (Fig. 4A, -), induced nuclear translocation in HMC cultured in a high-glucose medium. As NF-κB is the major transcription factor regulating a variety of inflammatory factors, including MCP-1 and ICAM-1 (6, 20, 29, 36, 42), we further investigated whether the inhibitory effects of PEDF on MCP-1 and ICAM-1 expression are through the inhibition of NF-κB activation. HMC were exposed to 30 mM glucose in the culture medium or the same concentration of mannitol as an osmolarity control for 48 h. PEDF was added into the medium at high glucose (Fig. 4A, -), induced nuclear translocation in HMC cultured in a high-glucose medium. As NF-κB is the major transcription factor regulating a variety of inflammatory factors, including MCP-1 and ICAM-1 (6, 20, 29, 36, 42), we further investigated whether the inhibitory effects of PEDF on MCP-1 and ICAM-1 expression are through the inhibition of NF-κB activation. HMC were exposed to 30 mM glucose in the culture medium or the same concentration of mannitol as an osmolarity control for 48 h. PEDF was added into the medium at high glucose (Fig. 4A, -), induced nuclear translocation in HMC cultured in a high-glucose medium. As NF-κB is the major transcription factor regulating a variety of inflammatory factors, including MCP-1 and ICAM-1 (6, 20, 29, 36, 42), we further investigated whether the inhibitory effects of PEDF on MCP-1 and ICAM-1 expression are through the inhibition of NF-κB activation. HMC were exposed to 30 mM glucose in the culture medium or the same concentration of mannitol as an osmolarity control for 48 h. PEDF was added into the medium at high glucose (Fig. 4A, -), induced nuclear translocation in HMC cultured in a high-glucose medium. As NF-κB is the major transcription factor regulating a variety of inflammatory factors, including MCP-1 and ICAM-1 (6, 20, 29, 36, 42), we further investigated whether the inhibitory effects of PEDF on MCP-1 and ICAM-1 expression are through the inhibition of NF-κB activation. HMC were exposed to 30 mM glucose in the culture medium or the same concentration of mannitol as an osmolarity control for 48 h. PEDF was added into the medium at high glucose (Fig. 4A, -), induced nuclear translocation in HMC cultured in a high-glucose medium. As NF-κB is the major transcription factor regulating a variety of inflammatory factors, including MCP-1 and ICAM-1 (6, 20, 29, 36, 42), we further investigated whether the inhibitory effects of PEDF on MCP-1 and ICAM-1 expression are through the inhibition of NF-κB activation. HMC were exposed to 30 mM glucose in the culture medium or the same concentration of mannitol as an osmolarity control for 48 h. PEDF was added into the medium at high glucose (Fig. 4A, -), induced nuclear translocation in HMC cultured in a high-glucose medium. As NF-κB is the major transcription factor regulating a variety of inflammatory factors, including MCP-1 and ICAM-1 (6, 20, 29, 36, 42), we further investigated whether the inhibitory effects of PEDF on MCP-1 and ICAM-1 expression are through the inhibition of NF-κB activation. HMC were exposed to 30 mM glucose in the culture medium or the same concentration of mannitol as an osmolarity control for 48 h. PEDF was added into the medium at high glucose (Fig. 4A, -), induced nuclear translocation in HMC cultured in a high-glucose medium. As NF-κB is the major transcription factor regulating a variety of inflammatory factors, including MCP-1 and ICAM-1 (6, 20, 29, 36, 42), we further investigated whether the inhibitory effects of PEDF on MCP-1 and ICAM-1 expression are through the inhibition of NF-κB activation. HMC were exposed to 30 mM glucose in the culture medium or the same concentration of mannitol as an osmolarity control for 48 h. PEDF was added into the medium at high glucose (Fig. 4A, -), induced nuclear translocation in HMC cultured in a high-glucose medium.
PEDF inhibits high glucose-induced overexpression of VEGF in HMC. To further determine the direct effect of PEDF on VEGF expression in the kidney, we have measured the VEGF secretion from HMC exposed to a high-glucose medium. The VEGF mRNA level was determined by quantitative real-time RT-PCR and expressed as percentage of that in nondiabetic control (mean ± SD; n = 4). B: VEGF protein levels in the kidney were measured by ELISA in diabetic rats 3 wk after adenovirus delivery. The results were normalized by total protein concentration in the kidney and expressed as pg/mg protein (mean ± SD; n = 4).

PEDF inhibits HIF-1α activation in HMC exposed to high glucose. HIF-1α is the major transcription factor regulating VEGF expression (17). To explore the mechanism underlying the inhibition of VEGF expression by PEDF, we examined the effect of PEDF on HIF-1α activation in HMC exposed to the high-glucose medium. In the cells exposed to the normal glucose medium, HIF-1α was predominantly distributed in the cytoplasm (Fig. 6, A–C). Incubation with mannitol did not change the distribution pattern of HIF-1α in HMC (Fig. 6, G–I). Exposure to the high-glucose medium for 24 h resulted in a translocation of HIF-1α from the cytoplasm to the nuclei, indicating an activation of HIF-1 induced by high glucose (Fig. 6, D–F). PEDF at 160 nM dramatically inhibited the high glucose-induced HIF-1α nuclear translocation (Fig. 6, J–L), indicating that PEDF inhibits HIF-1 activation induced by high glucose.

HIF-1α translocation was further quantified by an HIF-1α DNA-binding activity assay in the nuclear extracts from HMC. The results showed that activated HIF-1α was significantly increased by 1.5-fold in the HMC treated with high glucose compared with both of the untreated and mannitol controls (Fig. 6M, n = 3, P < 0.05). This increase was almost completely blocked by PEDF at 160 nM (Fig. 6, n = 3, P < 0.05).

DISCUSSION

PEDF is a member of the serpin superfamily. The neurotrophic functions of PEDF in the retinal neurons have been well established in animal models of inherited and light-induced retinal degeneration (5, 43). Most importantly, recent studies have shown that PEDF is a multifunctional protein with demonstrable neurotrophic, antitumorigenic, antiangiogenic, and antivasopermeability activities (8, 24, 43, 47). Our recent studies demonstrated that decreased PEDF levels in the kidney are associated with diabetic nephropathy, and Ad-PEDF delivery into STZ-diabetic rats significantly alleviated microalbuminuria in early stages of diabetes (46). In the present study, we extended these studies and determined whether the beneficial effects of PEDF on diabetic nephropathy can be ascribed to its anti-inflammatory activity. Our results showed that the Ad-PEDF treatment effectively blocked the overexpression of proinflammatory factors, including TNF-α, MCP-1, ICAM-1, and VEGF, in diabetic kidneys, correlating with the renal-protective effects of PEDF in diabetic kidneys. Our experiments showed that these effects are likely via inhibition of NF-κB and HIF-1 activation.

The roles of inflammation in diabetic nephropathy have been intensively studied (10, 12). It has been shown that TNF-α and IFN-γ are overexpressed in the kidneys of rats with long-term STZ-induced diabetes (16). In the same diabetic rat model,
Macrophages were recruited into the glomeruli at the very early phase of hyperglycemia in the glomeruli of rats with diabetes, and immunohistochemistry detected ED1 (monocyte/macrophage marker) in the glomeruli at day 8 of diabetes (16, 28, 33). In a kidney biopsy study conducted in diabetic patients, the number of macrophages and common leukocyte marker-positive cells increased significantly in the glomeruli at the moderate stage of glomerulosclerosis compared with the mild or advanced stage (12). Macrophages may transiently infiltrate during the moderate stage of diffuse diabetic glomerulosclerosis, contributing to irreversible structural damage (12). In ICAM-1-deficient (ICAM-1−/−) mice with experimental diabetes, the infiltration of macrophages was markedly suppressed compared with that in ICAM-1−/− mice with diabetes, suggesting that ICAM-1 plays an important role in inflammation in the diabetic kidney (32). Furthermore, urinary albumin excretion, glomerular hypertrophy, and mesangial matrix expansion were significantly lower in diabetic ICAM-1−/− mice than in diabetic ICAM-1−/− mice (32). These studies suggest that inflammation is critically involved in the pathogenesis of diabetic nephropathy.

Although PEDF has been shown to be a potent inhibitor of angiogenesis, it was recently found to have an anti-inflammatory function as well. Recent studies (47) by our group showed that retinal and plasma PEDF levels were drastically decreased in rats with endotoxin-induced uveitis. Intravitreal injection of PEDF significantly reduced vascular hyperpermeability in rat models of diabetes and oxygen-induced retinopathy, correlating with the decreased levels of retinal inflammatory factors, including VEGF, VEGF receptor-2, MCP-1, TNF-α, and ICAM-1 (47). In cultured retinal capillary endothelial cells, PEDF significantly decreased TNF-α and ICAM-1 expression under hypoxia. Moreover, downregulation of PEDF expression by
siRNA resulted in significant increases of VEGF and TNF-α secretion in retinal Müller cells (47). These findings suggest that PEDF is an endogenous anti-inflammatory factor in the eye. The present study provides further evidence showing that PEDF is also an endogenous anti-inflammatory factor in the kidney, and the decrease of renal PEDF levels in diabetic rat models may contribute to inflammation in the kidney.

Overexpression of intercellular adhesion molecules ICAM-1 (41), E-selectin and P-selectin, (37) and MCP-1 (3, 21) has been found to be involved in pathogenesis of diabetic nephropathy in diabetic patients and in animal models. TNF-α, which is produced mainly by monocytes and macrophages and also glomerular mesangial cells (4, 18), has been found to be increased in the kidney of diabetic animal models (16, 31). Although the mechanisms of upregulation for these molecules have not been defined, high ambient glucose levels have been suggested to be a major causative factor. Our present study showed that MCP-1, ICAM-1, and TNF-α expression in the kidneys of STZ rats was significantly increased compared with that in nondiabetic control rats. Treatment with PEDF has been shown to suppress renal MCP-1, ICAM-1, and TNF-α expression and to attenuate renal injury without altering glycemic control in diabetic rats. Taken together, these results suggest that PEDF has anti-inflammatory activities in the diabetic rat kidney, which may be responsible for the salutary effect of PEDF in diabetic nephropathy.

NF-κB is one of the most important transcription factors regulating ICAM-1 expression (14). On activation, NF-κB upregulates transcription of a number of target genes, including cytokines, adhesion molecules, nitric oxide synthases, and a variety of other inflammatory proteins involved in the pathogenesis of diabetic nephropathy (14). Our observation that PEDF can prevent nuclear translocation of NF-κB suggests a potential mechanism for the anti-inflammatory effect and renoprotective action of PEDF.

VEGF is strongly implicated in the pathogenesis of diabetic microvascular complications, as it increases vascular permeability to macromolecules and stimulates monocyte chemotaxis and tissue factor production, all of which can contribute to microvascular complications (9, 30). Although the role of VEGF in regulating glomerular permeability has not yet been defined, a growing body of clinical and experimental evidence.
suggests that VEGF be responsible for proteinuria in diabetic nephropathy (22, 23, 38). In the present study, Ad-PEDF treatment of rats with STZ-induced diabetes inhibited renal VEGF overexpression, which may contribute to the reduction of albuminuria after the PEDF treatment. This finding suggests that the effect of PEDF on albuminuria may be through blockade of VEGF expression in the diabetic kidney.

HIF-1α is a transcription factor that is increased in conditions of reduced cellular O2 (11, 34). The binding site of HIF-1α is present in the promoters of several genes, such as VEGF, that are sensitive to changes in Po2 (27, 35). A recent report (7) suggested that HIF-1α could also be induced during inflammation. Inhibiting HIF-1α translocation by PEDF could be another important mechanism for the anti-inflammatory activities of PEDF in diabetic nephropathy. It is not clear whether the inhibitory effect of PEDF on HIF-1 activation is related to or independent of the inhibition of NF-κB.

In summary, our data suggest that PEDF is an endogenous anti-inflammatory factor in the kidney, and decreased PEDF levels in diabetic kidney may contribute to the inflammation in the kidney, subsequently leading to diabetic nephropathy. The salutary effect of PEDF on diabetic nephropathy may be mediated, at least in part, by its anti-inflammatory activity. Therefore, PEDF treatment may become an anti-inflammatory therapy in diabetic nephropathy.

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