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Role of vascular endothelial growth factor in diabetic nephropathy

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Role of vascular endothelial growth factor in diabetic nephropathy.

Background. Vascular endothelial growth factor (VEGF) is a potent cytokine that is considered to be an important mediator in the pathogenesis of endothelial dysfunction in diabetes.

Methods. This study investigates the effect of high glucose on the signaling and production of VEGF in rat mesangial cells in culture and measures the urinary VEGF level in patients with different stages of diabetic nephropathy. To elucidate the role of VEGF in vivo further, expression of VEGF in control and diabetic kidneys was examined using immunohistochemistry.

Results. A high ambient glucose concentration in the culture medium increased VEGF mRNA expression and protein production within 3 h in a concentration-dependent manner. A protein kinase C (PKC) inhibitor and PKC down-regulation inhibited glucose-induced increases in VEGF production. Urinary excretion of VEGF significantly increased according to the degree of proteinuria in patients with diabetes. A weak but significant correlation was found between urinary VEGF excretion and the levels of serum creatinine, creatinine clearance, microalbuminuria, and proteinuria. Immunohistochemistry revealed marked differences in the extent of mesangial VEGF staining between diabetic and control kidneys. Pronounced up-regulation of VEGF was observed in the glomerular epithelial cell in the early phase of diabetic kidney disease, whereas widespread expression of VEGF was found in the tubular segments, especially the proximal segment, in advanced diabetic nephropathy.

Conclusions. These results suggest that VEGF may play a role in the pathogenesis of diabetic nephropathy.

Abnormalities in endothelial function, such as increased endothelial permeability to macromolecules and endothelial proliferation, may be involved in the pathogenesis of diabetic microvascular complications [1]. Considerable research has focused on the pathogenesis of endothelial dysfunction, but the exact mechanisms remain incompletely defined.

Vascular endothelial growth factor (VEGF) is an attractive candidate in this regard because VEGF markedly increases vascular permeability and potently stimulates the vascular endothelial cell to divide [2, 3]. Its pathogenic role in diabetic retinopathy is widely accepted, and it predominantly mediates the neoangiogenesis associated with proliferative retinopathy [4, 5]. Endothelial dysfunction, which is also associated with diabetic nephropathy, may play a pathogenic role in the development of diabetic renal disease [6, 7], but this remains speculative at this time.

In this study, we examined the effects of a high-glucose concentration in culture media on VEGF mRNA expression and protein production in rat mesangial cells. We also established whether VEGF production depends on the protein kinase C (PKC) pathway. In a human study, we determined the plasma and urine concentrations of VEGF and searched for any relationship between these values and the severity of diabetic nephropathy. To understand the role of VEGF in diabetic kidney disease further, renal biopsies from 20 patients with noninsulin-dependent diabetes mellitus (NIDDM) and 3 patients with renal cell carcinoma undergoing nephrectomy were stained with a rabbit anti-VEGF antibody.

METHODS

Rat mesangial cell culture

Glomeruli were isolated from male Sprague-Dawley rats weighing an average of 100 g. The kidney was removed and the glomeruli, isolated by differential sieving, were incubated in collagenase and trypsin-EDTA (Gibco Laboratories, Bethesda, MD, USA), as described previously [8]. Mesangial cells (MCs) were identified by their characteristic stellate shape in cell culture and confirmed by immunofluorescent microscopy for the presence of actin and myosin and the absence of factor VIII and cytokeratin (Synbiotics, San Diego, CA, USA). MCs were maintained in normal glucose (5.5 mmol/L) Dulbecco's Modified Eagle Medium (DMEM) supplemented

Key words: cytokines, diabetes mellitus, proteinuria.

with 2 mmol/L L-glutamine, 10 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 100 U/mL penicillin, 100 U/mL streptomycin, and 17% heat-inactivated fetal bovine serum (FBS). For this study, cells in the fourth to sixth passages were incubated at 37°C in humidified 5% CO_2 in air.

Experimental design

MCs were grown to subconfluence in 6-well plates in the growth media, and then cultured for 48 hours in serum-deprived medium containing 5 mmol/L D-glucose and 1% FBS, before being exposed to an experimental condition. Cells were divided into three experimental groups according to the glucose concentration: The normal glucose group (NG), served as control; the medium glucose group (MG) was incubated in 15 mmol/L D-glucose; and the high glucose group (HG) was incubated in 30 mmol/L D-glucose. As an osmotic control, MCs were cultured in media containing 5 mmol/L D-glucose and 25 mmol/L D-mannitol. MCs grown in triplicate were harvested at 0, 3, 6, and 24 hours for extraction of total RNA and protein. To evaluate whether VEGF production depends on the protein kinase C pathway, MCs were treated with 200 nmol/L calphostin C (Calbiochem, San Diego, CA, USA) for 60 minutes before changing to the 30 mmol/L D-glucose media. To determine the effect of PKC down-regulation, MCs were treated with 100 nmol/L phorbol 12-myristate 13-acetate (PMA, Sigma Chemical Corporation, St. Louis, MO, USA) for 24 hours, incubated in 30 mmol/L D-glucose for 3 hours, and harvested for total RNA and protein extraction. To avoid any confounding effects of serum on VEGF production, all experiments were performed in serum-free media.

Semiquantitative RT-PCR of VEGF transcripts

Total RNA was extracted from MCs using TRIzol LS reagent (Life Technologies, Gaithersburg, MD, USA) and the cDNA was synthesized by a reverse transcription reaction of 1 μg of total RNA with oligo-(dT) primers (Life Technologies, Gaithersburg, MD, USA). Next, 5 μL cDNA was amplified by Taq DNA polymerase in a 25-μL reaction volume containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L deoxyribonucleoside triphosphate, and 10 pmol of each primer. The polymerase chain reaction (PCR) profile consisted of initial denaturation at 94°C for 7 minutes, followed by 35 cycles (VEGF) or 30 cycles (β_2-microglobulin [β_2m]) of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 90 seconds, and extension at 72°C for 7 minutes.

Two sets of rat VEGF primers, which amplify at least three splicing variants of rat VEGF mRNA ($VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$), were used. The first set (rVEGF1 primer), which includes introns between amplification sites from exon 3 to the 3′ untranslated end, amplifies three splicing variants ($VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$). The expected lengths of their PCR products are 330 base pairs (bp) for $VEGF_{120}$, 462 bp for $VEGF_{164}$, and 514 bp for $VEGF_{188}$. The nucleotide sequences of each primer are as follows: sense 5′-GACCCTGGTGGACATCTTC CAGGA-3′ and antisense 5′-GGTGAGAGGTCTAG TTCCCGA-3′. The second set (rVEGF2 primer), which includes introns between amplification sites from exon 3 to exon 7, amplifies two splicing variants ($VEGF_{164}$ and $VEGF_{188}$). The expected lengths of their PCR products are 444 bp for $VEGF_{164}$ and 372 bp for $VEGF_{188}$. The nucleotide sequences of each primer are as follows: sense 5′-GACCCTGGTGGACATCTTCCAGGA-3′ and antisense 5′-GTTTAACTCAAGCTGCCTCGC-3′. β_2m was also amplified as an internal control, and the expected length of the PCR product was 188 bp. The nucleotide sequences of the primers are as follows: sense 5′-CAGA TCTGTCCTTCAGCAAG-3′ and antisense 5′-GGAGT AAACTGGTCCAGATG-3′.

Different numbers of PCR cycles were performed and plotted against the densitometry measurements of the resulting PCR products to define the range in which cycle number was linearly related to the amount of PCR-amplified product. Next, 5 μL PCR reaction product were separated by electrophoresis through a 2% agarose gel with ethidium bromide. PCR product bands, visualized by ultraviolet light, were scanned at 300 dots per inch and densitometric analysis was done with the National Institutes of Health Image 1.61 software with available macro and linear regression analyzer. To confirm the identity of the VEGF cDNA product, each of the electrophoresed PCR bands was extracted with a DNA extraction kit (Qiagen, Valencia, CA, USA) and sequenced using an ABI automated DNA sequencing system (ABI Genetic Analyzer 310; PRISM, Branchburg Park, NJ, USA). Quantitation of the VEGF mRNA expression was corrected by β_2m.

Measurement of VEGF protein using Western blot

After incubation in the different culture media, MCs were lyzed in lysis buffer and centrifuged for 15 minutes at 12,000 rpm. Next, 50 μg protein was electrophoresed on 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The blot was incubated at room temperature on a shaker mechanism in 0.1 ng/μL of an anti-VEGF monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Detection of specific signals was performed using the ECL Western blotting detection reagents according to the manufacturer's recommendations (Amersham, Buckinghamshire, England).

Measurement of VEGF concentrations in plasma and urine of patients with diabetes

Seventy-three patients with NIDDM whose serum creatinine was less than 2.0 mg/dL were included for mea-

surement of VEGF levels in plasma and urine according to the status of diabetic nephropathy. The amount of VEGF protein was measured by a competitive enzyme immunoassay (EIA) kit (ACCUCYTE Human VEGF; Cytimmune Science Inc., MD, USA). At that point, 50 μL of a plasma or urine sample with an equal volume of diluent was dispensed in a 96-microwell plate, precoated with 25 μL diluted biotinylated rabbit antihuman VEGF polyclonal antibody. The plate was incubated at room temperature for 3 hours, washed five times, and developed with 200 μL of color reagent per well. The intensity of the color was measured in an enzyme-linked immunosorbent assay (ELISA) reader. The sensitivity of the ELISA for VEGF was 19.5 pg/mL.

Immunohistochemistry

This study was performed to analyze in detail the expression of VEGF protein in the diabetic kidney. Twenty renal biopsies from patients with NIDDM and three biopsies from patients with renal cell carcinoma undergoing nephrectomy were studied. In the group with diabetes, the average age of the patients was 50.9 ± 14 years (mean ± standard deviation [SD]); the average daily excretion of urine protein was 2.55 ± 1.88 g (mean ± SD); and the average serum creatinine concentration was 1.56 ± 1.12 mg/dL (mean ± SD). Renal tissue was obtained from percutaneous biopsy in patients with NIDDM and from tumor-free parts of the nephrectomy specimen in patients with renal cell carcinoma. Renal tissue was immediately fixed in 10% neutral buffered formalin, cast in paraffin, sliced in 3 μm thick sections, and placed on a microscope slide. After removal of paraffin and dehydration in xylene and graded alcohols, the slide was immersed in distilled water. The kidney section was transferred to a 10-mmol/L citrate buffer solution for antigen retrieval at a pH of 6.0 and then microwaved for 10 min. After another water wash, 0.05% H_2O_2-methanol was applied for 15 minutes to block the endogenous peroxidase. The primary antibody, polyclonal anti-VEGF (Biogenex, San Ramon, CA, USA), was added at a 1:20 dilution for 2 hours at room temperature. Negative control sections were stained under identical conditions with the buffer solution substituting for the primary antibody. With an LSAB kit/HRP (DAKO, Carpinteria, CA, USA), kidney sections were sequentially treated with normal goat serum, primary antibody, link antibody, streptavidin-biotin horseradish peroxidase, and aminoethyl carbamisole (chromogen). Sections were then counterstained with Mayer's hematoxylin.

Statistics

Results were expressed as mean ± SD. Comparisons were made by analysis of variance (ANOVA) within groups and by Student's *t*-test between groups using a SPSS for Windows computer application. To analyze the correlation between urinary VEGF and various clinical parameters, simple regression analysis was used. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of various glucose concentrations on VEGF gene expression

RT-PCR of mRNA from rat MCs using rVEGF1 primers showed three alternative splicing variants: bands of 330, 462, and 514 bp corresponding to $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$, respectively (Fig. 1). Likewise, RT-PCR amplification using rVEGF2 primers revealed two alternative splicing variants: 377 and 439 bp fragments corresponding to $VEGF_{164}$ and $VEGF_{188}$, respectively (Fig. 1).

We then addressed the effect of increasing glucose concentrations in the culture media on the level of the VEGF transcript. In the normal glucose group, low levels of VEGF isoforms were expressed, and the ratio of $VEGF_{164}$ to β_2m was 0.03 ± 0.03. However, mRNA expression of VEGF was incrementally increased in a glucose concentration–dependent manner. In the medium glucose (15 mmol/L D-glucose) group, the ratio of $VEGF_{164}$ to β_2m was increased to 0.12 ± 0.07 at 3 hours, 0.05 ± 0.02 at 6 hours, and 0.03 ± 0.01 at 24 hours. The $VEGF_{164}$ to β_2m ratio in the high glucose (30 mmol/L D-glucose) group was markedly increased to 0.48 ± 0.10 at 3 hours ($P = 0.016$), 0.25 ± 0.08 at 6 hours ($P = 0.044$), and 0.04 ± 0.05 at 24 hours (Fig. 2, Table 1).

The $VEGF_{120}$ to β_2m ratio was similar to that of $VEGF_{164}$. In the medium glucose group, it was increased to 0.17 ± 0.15 at 3 hours ($\mu L = 0.03$) and 0.09 ± 0.03 at 6 hours ($\mu L = 0.043$). In the high glucose group, it was increased to 0.57 ± 0.10 at 3 hours ($\mu L = 0.012$) and 0.48 ± 0.08 at 6 hours ($\mu L = 0.014$) (Fig. 2, Table 2). MCs exposed to 25 mmol/L D-mannitol for 3 hours showed no difference in the expression of VEGF mRNA compared with that found in the normal glucose group.

Effect of PKC inhibition on VEGF gene expression

To test whether VEGF expression is mediated by the PKC system, PMA and calphostin C were used. Treatment with calphostin C (200 nmol/L) for 1 hour before incubation in high-glucose media markedly decreased $VEGF_{164}$ expression to 0.09 ± 0.06 at 3 hours ($P = 0.04$) and 0.03 ± 0.03 at 6 hours ($P = 0.071$) compared with no calphostin C. Similarly, $VEGF_{120}$ expression was also decreased to 0.13 ± 0.07 at 3 hours ($P = 0.003$) and 0.05 ± 0.03 at 6 hours ($P = 0.014$). Treatment of MCs with PMA (100 nmol/L) for 24 hours in high-glucose media prevented the high glucose–induced increases in $VEGF_{164}$ expression (0.08 ± 0.05; $P = 0.034$) and $VEGF_{120}$ expression (0.06 ± 0.05; $P = 0.035$) (Fig. 3, Tables 1 and 2).

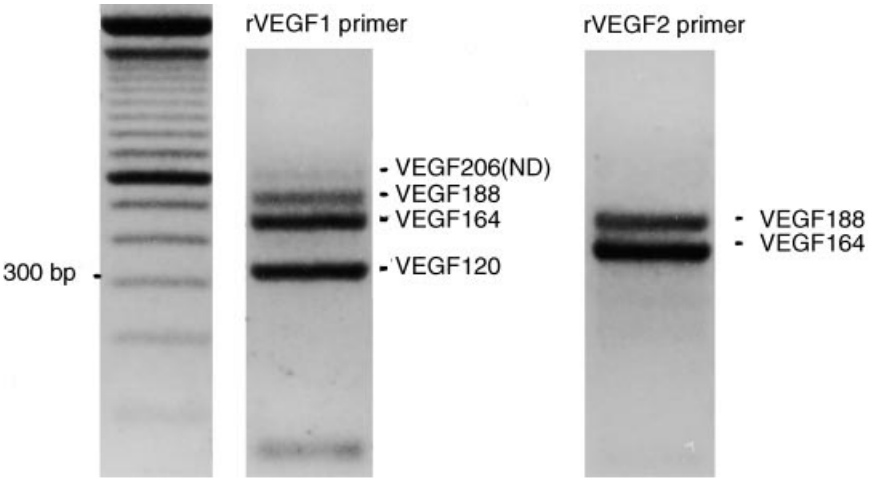

Fig. 1. RT-PCR of VEGF in cultured rat mesangial cells. The three alternative splicing variants encoding peptides $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$ were identified using rVEGF1 primers. Two splicing variants, $VEGF_{164}$ and $VEGF_{188}$, were identified using rVEGF2 primers. $VEGF_{120}$ and $VEGF_{164}$ were expressed more highly than $VEGF_{188}$, which was faintly expressed.

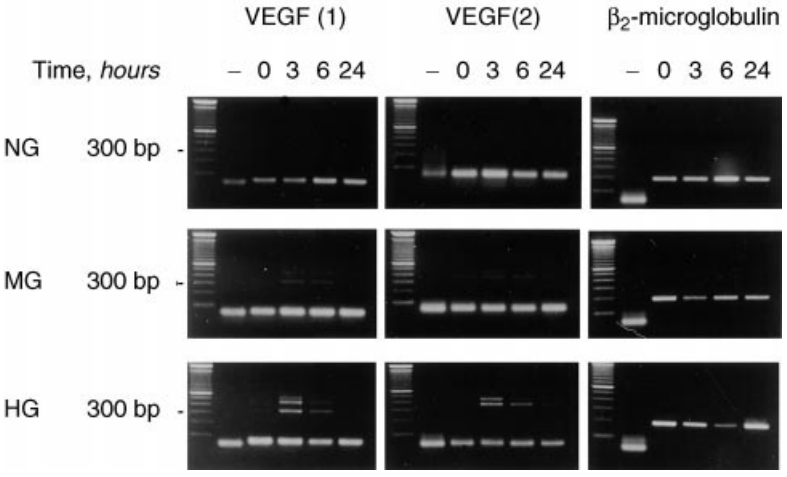

Fig. 2. The effect of glucose concentration on mRNA expression of VEGF. VEGF expression was increased in high glucose media and reached a peak value in 30 mmol/L D-glucose at 3 hours' incubation. The expression of $VEGF_{120}$ and $VEGF_{164}$ was higher than that of $VEGF_{188}$. NG, normal glucose; MG, medium glucose; HG, high glucose

Table 1. $VEGF_{164}$ mRNA expression in each experimental condition

Glucose or treatment group	Hours of incubation			
	0	3	6	24
NG	0.03 ± 0.03	—	—	—
MG	—	0.12 ± 0.07	0.05 ± 0.02	0.03 ± 0.01
HG	—	0.48 ± 0.10*	0.25 ± 0.08*	0.04 ± 0.05
Cal	—	0.09 ± 0.06†	0.03 ± 0.03	—
PMA	—	0.08 ± 0.05†	—	—

Results are shown as $VEGF_{164}/\beta_2$m mRNA densitometric recording ratio. Data are expressed as mean ± SD. NG, normal glucose group; MG, medium glucose group; HG, high glucose group; Cal, calphostin C-treated group; PMA, phorbol myristate acetate-treated group.
* $P < 0.05$ compared with normal glucose group at 0 hour incubation.
† $P < 0.05$ compared with high glucose group at 3 hours of incubation.

Table 2. $VEGF_{120}$ mRNA expression in each experimental condition

Glucose or treatment group	Hours of incubation			
	0	3	6	24
NG	0.04 ± 0.03	—	—	—
MG	—	0.17 ± 0.15*	0.19 ± 0.03*	0.03 ± 0.02
HG	—	0.57 ± 0.10*	0.48 ± 0.08*	0.04 ± 0.04
Cal	—	0.13 ± 0.07†	0.05 ± 0.03†	—
PMA	—	0.06 ± 0.05†	—	—

Results are shown as $VEGF_{120}/\beta_2$m mRNA densitometric recording ratio. Data are expressed as mean ± SD. NG, normal glucose group; MG, medium glucose group; HG, high glucose group; Cal, calphostin C-treated group; PMA, phorbol myristate acetate-treated group.
* $P < 0.05$ compared with normal glucose group at 0 hour incubation.
† $P < 0.05$ compared with high glucose group at 3 hours of incubation.

Western blot analysis of VEGF production in cultured MCs

VEGF protein in MCs was detected as a single band of approximately 42 kDa. Similar to the findings with VEGF mRNA, VEGF protein expression remained low in the normal glucose group but increased in a glucose concentration–dependent manner, peaking at 3 hours. Likewise, pretreatment with PMA or calphostin C markedly decreased production of VEGF protein in the high-glucose group. Moreover, D-mannitol had no effect on VEGF protein production at 3 h (Fig. 4).

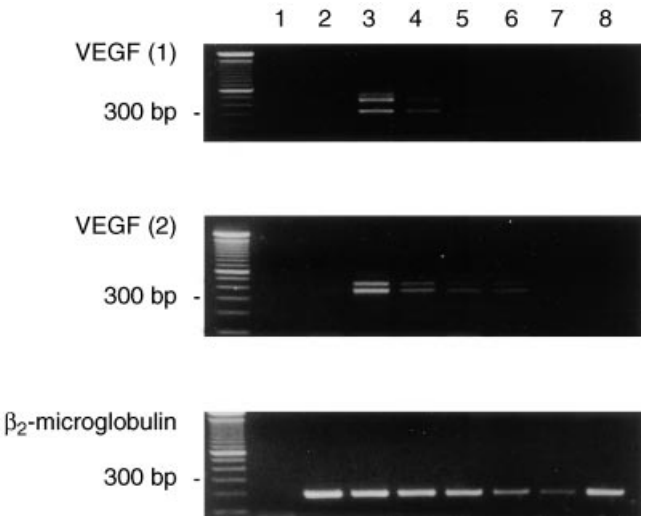

Fig. 3. The effect of PKC inhibitor (calphostin C) and PKC down-regulator (PMA) on VEGF mRNA expression. VEGF mRNA expression was significantly decreased with calphostin C and PMA treatment. Mannitol had no effect on VEGF expression. 1: negative control, 2: normal glucose at 0 hour, 3: high glucose at 3 hours, 4: calphostin C in high glucose at 3 hours, 5: calphostin C in high glucose at 6 hours, 6: calphostin C in high glucose at 24 hours, 7: PMA in high glucose at 3 hours, and 8: mannitol at 3 hours.

VEGF concentration in plasma and urine in patients with diabetes

In the study, 73 patients with NIDDM whose serum creatinine level was less than 2.0 mg/dL were included. Patients were classified according to the status of their diabetic nephropathy (37 cases with normoalbuminuria, 23 with microalbuminuria, and 13 with overt proteinuria). Baseline clinical and biochemical characteristics of the study population are shown in Table 3. Plasma VEGF concentration was significantly higher in the patients with overt proteinuria (1219.2 ± 463.9 pg/mL) than in patients with normoalbuminuria (937.0 ± 429.7 pg/mL) and microalbuminuria (857.8 ± 455.6 pg/mL) ($P < 0.05$). Plasma VEGF level did not differ between patients with normoalbuminuria and those with microalbuminuria.

Urinary excretion of VEGF was significantly increased according to the degree of microalbuminuria and proteinuria. Urinary VEGF concentration was 57.2 ± 87.9 pg/mg Cr in normoalbuminuric patients, 122.7 ± 75.1 in microalbuminuric patients, and 288.4 ± 119.3 in overtly proteinuric patients ($P < 0.05$). The level of urinary VEGF was also weakly but significantly correlated with the levels of serum creatinine ($r = 0.389$; $P = 0.001$), microalbuminuria ($r = 0.308$; $P < 0.05$), proteinuria ($r = 0.491$; $P < 0.0001$), and creatinine clearance ($r = -0.327$; $P < 0.05$). In a multiple regression analysis, the 24-hour urine protein excretion and serum creatinine concentration were independently associated with the urinary VEGF level.

Immunohistochemical staining for VEGF in the diabetic kidney

In the normal kidney, VEGF protein was clearly detected in the glomerular visceral epithelial cells, evidenced by strong cytoplasmic staining, but not in mesangial and endothelial cells (Fig. 5A). In the renal tubule compartment, all positively stained cells showed a cytoplasmic pattern (Fig. 5B), which was focally increased in the distal tubular epithelial cells and faint in the proximal tubular cells. VEGF staining was markedly different in the diabetic vs. the normal kidney with changes primarily localized in the glomerular mesangium. Biopsy results showing mild changes of diabetic nephropathy stained more heavily for VEGF in the glomerular visceral epithelial and distal tubular cells than those of control kidneys (Fig. 5C). In contrast, the biopsy results showing advanced changes of diabetic nephropathy, such as severe mesangial sclerosis and mesangial expansion, showed markedly decreased and sometimes negative VEGF staining in globally sclerotic glomeruli (Fig. 5D). Interestingly however, VEGF staining in the tubule compartment, particularly the proximal segment, remained more intense in the biopsies of patients with late diabetic nephropathy than in those of early nephropathy (Fig. 5E).

DISCUSSION

We demonstrate that the rat mesangial cell cultured in high-glucose media transiently up-regulates its expression of VEGF mRNA and protein, which arise as three alternative splicing variants. This finding agrees with a previous report that showed that human mesangial cells are capable of producing VEGF [9]. Williams et al [10] reported that vascular smooth muscle cells grown in high-glucose media overexpress VEGF through the PKC pathway. Considering that MCs share properties with smooth muscle cells, this raises the possibility that MCs can produce VEGF through a PKC-dependent mechanism.

The MC is apposed to the glomerular capillary endothelial cell, which possesses receptors for VEGF [11, 12]. This anatomic relationship may allow the locally produced VEGF by MCs to directly influence glomerular endothelial cell function. Diabetes results in several pathobiologic changes, such as activation of PKC, up-regulation of cytokines and growth factors including transforming growth factor (TGF)-β, and stimulation of the renin-angiotensin system [13–19], which are all known to increase renal VEGF production. Therefore, the mesangial cell in the diabetic state may be plausibly expected to augment its production of VEGF. In the present study, VEGF expression peaked at 3 hours in the high-glucose group, and VEGF production was increased in a glucose concentration–dependent manner. This finding is similar to that of a study in which VEGF production peaked at 3 hours in vascular smooth muscle cells cultured in high-glucose solution [10].

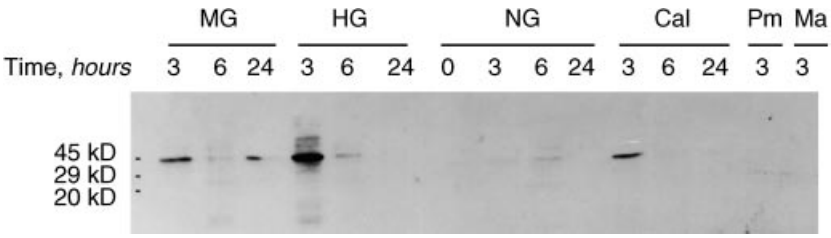

Fig. 4. Western blot analysis of VEGF production. Distinct bands of VEGF dimers were detected at approximately 42 kDa. The VEGF peptide was increased in high glucose media, particularly at 3 hours' incubation. VEGF production was markedly suppressed in the calphostin C- or PMA-treated group. NG, normal glucose; MG, medium glucose; HG, high glucose; Cal, calphostin C-treated group; P, PMA-treated group; M, mannitol-treated group.

Table 3. Baseline clinical and biochemical characteristics of the study population

Parameter	Normo-albuminuria	Micro-albuminuria	Overt proteinuria
Age *years*	51.3 ± 17.8	53.6 ± 13.8	57.3 ± 8.8
Systolic BP *mm Hg*	122.4 ± 14.3	131.9 ± 16.6	143.3 ± 21.9‡
Diastolic BP *mmHg*	79.7 ± 7.5	81.1 ± 7.6	83.3 ± 7.8
Hb_{A1C} *%*	8.6 ± 2.2	8.9 ± 2.0	8.5 ± 1.7
BUN *mg/dL*	15.2 ± 5.8	23.9 ± 31.9	26.7 ± 19.7
Creatinine *mg/dL*	0.78 ± 0.25	0.78 ± 0.20	1.62 ± 0.33‡
Cholesterol *mg/dL*	181 ± 40	180 ± 46	190 ± 51
Albumin *g/dL*	4.18 ± 0.46	4.04 ± 0.59	3.36 ± 0.60‡
C_{Cr} *mL/min*	131 ± 47	133 ± 51	55 ± 29‡
24 h proteinuria *mg/day*	293 ± 206	541 ± 399	4400 ± 3411‡
Microalbuminuria *mg/day*	12.6 ± 6.9	107.3 ± 81.1*	1043 ± 998‡
Plasma VEGF *pg/mL*	949 ± 439	861 ± 478	1215 ± 484
Urine VEGF *pg/mg Cr*	60.2 ± 89.5	128.7 ± 75.4†	286.0 ± 124.3‡

Results are given as mean ± standard deviation. Comparisons are made between the normoalbuminuric group, microalbuminuric group, and the overt proteinuric group. Number of patients is 37 in the normoalbuminuric group, 23 in the microalbuminuric group, and 13 in the overt proteinuric group. Statistically significant difference vs. normoalbuminuric patients: $*P < 0.05$, $\dagger P < 0.001$, $\ddagger P < 0.0001$

That high ambient glucose causes an early, but not sustained, release of VEGF in vitro seems to argue against a role for VEGF in diabetic nephropathy, but prolonged hyperglycemia and other consequences of the diabetic state in vivo may alter the relative balance among various growth factors and cytokines such that VEGF production may be sustained for a longer period [20–23]. In the present study, high glucose–induced VEGF synthesis was abolished by PKC inhibition or down-regulation, which suggests that PKC pathways are involved in VEGF production in MCs. It is thus possible that a PKC inhibitor may help to prevent overproduction of VEGF in diabetes.

Consistent with a previous study [9], RT-PCR results revealed that rat MCs express mRNA splicing variants corresponding to the $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$ isoforms. All three transcripts, especially those of $VEGF_{120}$ and $VEGF_{164}$, appear to be up-regulated by high-glucose media. $VEGF_{120}$ and $VEGF_{164}$ are secreted isoforms that bind with high affinity to the vascular endothelium through two specific tyrosine kinase receptors [24]. Although differences in the biologic activities of these different splicing variants have been reported, it remains unclear whether they perform different biologic functions in vivo [12]. Alternative splicing of VEGF mRNA occurs in a tissue-specific manner, and the relative abundance of each variant differs among the tissue types [12, 25]. In the kidney, $VEGF_{164}$ is the most abundant product of the VEGF gene, but in this study, glucose-induced expression of $VEGF_{120}$ was slightly higher than that of $VEGF_{164}$ in rat MCs.

VEGF is strongly implicated in the pathogenesis of diabetic microvascular complications because it increases vascular permeability to macromolecules and stimulates monocyte chemotaxis and tissue factor production, all of which can contribute to microvascular complications [26, 27]. Although the role of VEGF in regulating glomerular permeability has not yet been defined, it can potentially mediate glomerular proteinuria in diabetic nephropathy. The mechanism of increased vascular permeability by VEGF may involve stimulation of collagenase production and proteolytic disruption of the endothelial basement membrane [28]. Antonetti et al [29, 30] reported that vascular permeability in experimental diabetes is associated with reduced endothelial occludin, a tight junction protein between endothelial cells.

With regard to vascular permeability, Williams et al [abstract; *Diabetes* 45(Suppl 2):66A, 1996] showed that an acute infusion of VEGF into experimental animals markedly increased sciatic nerve and aortic albumin permeability. It is tempting to speculate that the VEGF produced by MCs may act as a paracrine factor to influence glomerular protein permselectivity.

In this study, urinary excretion of VEGF in people with type 2 diabetes was significantly increased with progression from microalbuminuria to overt proteinuria and was correlated with the levels of serum creatinine and the degree of proteinuria. Whether the increase in urinary VEGF excretion is a primary cause or a secondary effect of diabetic nephropathy remains unanswered.

It has been reported that the kidney, particularly the glomerulus, is a major source of VEGF production in humans [31, 32]. This study showed that the VEGF protein is localized primarily in the glomerular epithelial cells and to a lesser extent in the distal tubular epithelial cells in the normal kidney, consistent with the findings of

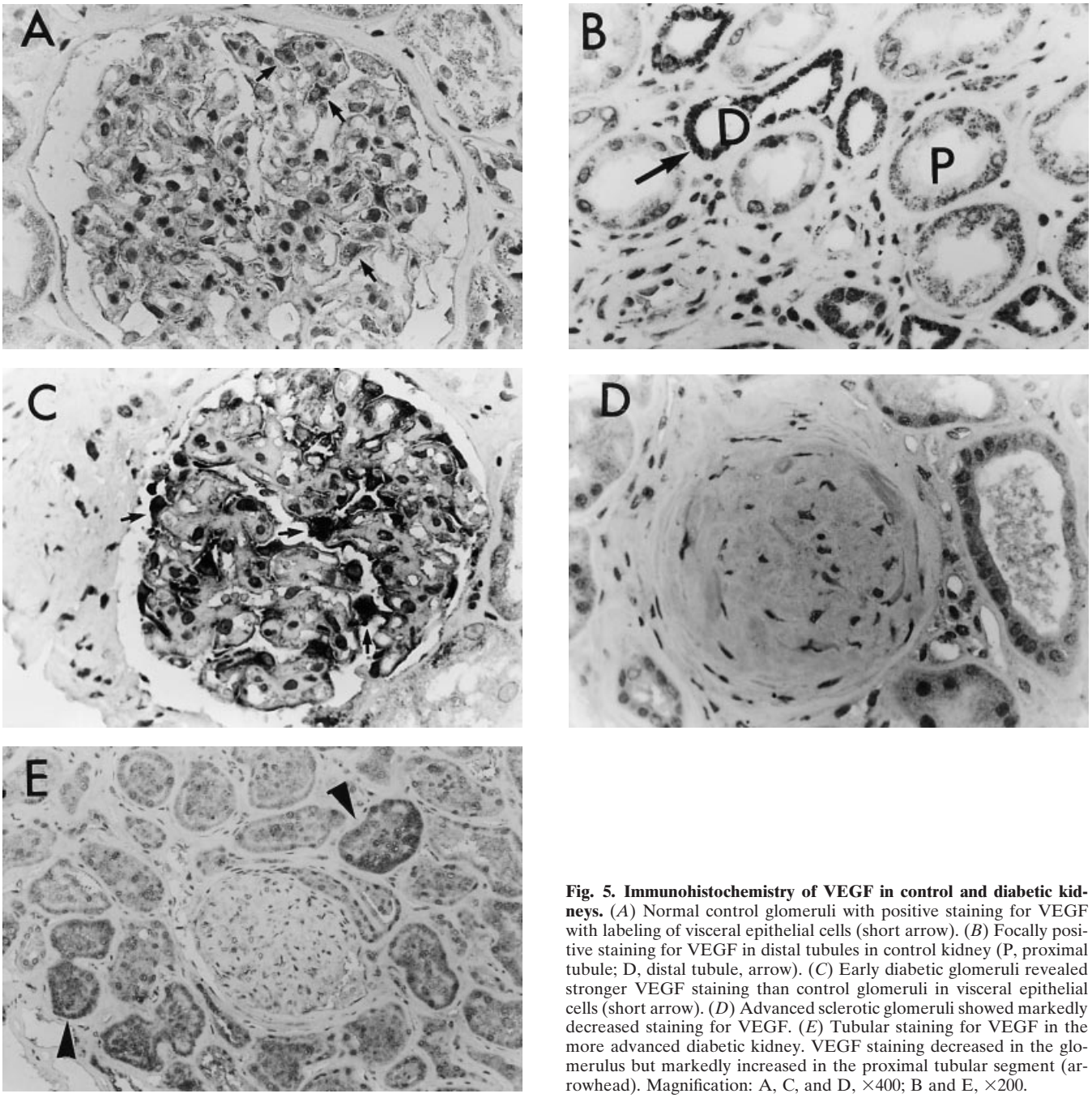

Fig. 5. Immunohistochemistry of VEGF in control and diabetic kidneys. (*A*) Normal control glomeruli with positive staining for VEGF with labeling of visceral epithelial cells (short arrow). (*B*) Focally positive staining for VEGF in distal tubules in control kidney (P, proximal tubule; D, distal tubule, arrow). (*C*) Early diabetic glomeruli revealed stronger VEGF staining than control glomeruli in visceral epithelial cells (short arrow). (*D*) Advanced sclerotic glomeruli showed markedly decreased staining for VEGF. (*E*) Tubular staining for VEGF in the more advanced diabetic kidney. VEGF staining decreased in the glomerulus but markedly increased in the proximal tubular segment (arrowhead). Magnification: A, C, and D, ×400; B and E, ×200.

previous reports [33, 34]. One can speculate that VEGF, constitutively produced by the podocyte, may play some role in regulating the glomerular permeability to protein.

VEGF staining in the diabetic kidney demonstrated marked differences with respect to degree of the glomerular mesangial expansion. Interestingly, biopsies of patients who had experienced mild changes of diabetic nephropathy showed greater VEGF staining in the glomerulus than biopsies of control specimens. Although this does not establish causality, up-regulation of VEGF may contribute to the early changes of diabetic nephropathy. However, the small number of patients in this study with an early diabetic lesion precludes any definitive conclusions about the role of VEGF in this stage of the disease.

Pathologic findings differed somewhat from those of urinary VEGF levels. Even though VEGF staining in the glomerulus decreased with worsening of diabetic nephropathy, VEGF excretion in the urine increased with progression of proteinuria. Patients with early diabetic nephropathy, evidenced by microalbuminuria, may pro-

duce most of their VEGF from glomerular visceral epithelial cells. In contrast, patients with more advanced nephropathy, evidenced by global sclerosis and overt proteinuria, may produce much of their VEGF in the tubules, especially proximally, which demonstrated a marked up-regulation of VEGF staining. It is possible that as nephropathy progresses, increased tubular epithelial VEGF production more than compensates for decreased glomerular VEGF production.

The renal tubule is subject to both direct and indirect pathogenetic influences because of its position in the nephron and its reabsorptive function [35–37]. In the diabetic state, the tubule, particularly the proximal segment, is directly exposed to glomerular effluents including glucose, large amounts of protein, advanced glycosylation end products, and other substances. Many of these activate various cytokines and growth factors including TGF-β and VEGF.

Conversely, tubular ischemia can result from increased metabolic demand on the tubules or reduced peritubular blood flow [38]. In this regard, the S3 segment of the proximal tubule may be particularly vulnerable to ischemia, which is a potent inducer of VEGF production [39]. Additional studies will be needed to formally address these issues.

In summary, high-glucose concentration in culture media transiently increases VEGF mRNA expression and protein production in rat MCs through a PKC-dependent mechanism. Urinary excretion of VEGF significantly increases according to the stage of diabetic nephropathy, and it also weakly but significantly correlates with the level of serum creatinine, creatinine clearance, and degree of proteinuria. Pronounced up-regulation of VEGF is found in the glomerular epithelial cell in early diabetic nephropathy, whereas a marked increase in VEGF is found in the proximal tubular cell in more advanced nephropathy.

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