CLINICAL STUDY

Quantitative and qualitative changes in vascular endothelial growth factor gene expression in glomeruli of patients with type 2 diabetes

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Abstract

Objective: Vascular endothelial growth factor (VEGF) exists in three main splice variants, characterized by 121, 165 and 189 amino acids (VEGF 121, VEGF 165 and VEGF 189) and acts via two specific receptors: VEGF-R1 or Flt-1 and VEGF-R2 or KDR. VEGF plays an important role in the pathogenesis of diabetic retinopathy. This study examined the relationship between VEGF and its isoforms and the severity of diabetic nephropathy in type 2 diabetes.

Design: We evaluated the glomerular gene expression of VEGF and its receptors and studied the relationships with renal functional and structural parameters in type 2 diabetic patients.

Methods: Glomeruli from 17 kidney biopsies were microdissected; 14 out of 17 biopsies were also subjected to electron microscopic morphometric analysis to estimate glomerular structural parameters. VEGF mRNA was studied by comparative kinetic RT-PCR and real-time RT-PCR in order to identify the three different isoforms and to quantify VEGF, VEGF-R1 and VEGF-R2 mRNA levels.

Results: (i) Glomerular VEGF mRNA levels were inversely related to albumin excretion rate ($r = -0.66$, $P = 0.004$); (ii) both the degree of mesangial and mesangial matrix expansion were inversely related to VEGF 165 mRNA levels ($r = -0.73$, $P = 0.005$ and $r = -0.64$, $P = 0.017$), and directly to VEGF 121 mRNA levels ($r = 0.74$, $P = 0.003$ and $r = 0.73$, $P = 0.004$); and (iii) VEGF and VEGF-R2 mRNA levels were directly related ($r = 0.62$, $P = 0.033$).

Conclusions: These findings suggested that quantitative and qualitative changes in VEGF expression are present in type 2 diabetic patients with nephropathy and might be involved in the pathogenesis and progression of diabetic glomerulopathy.

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Introduction

Vascular endothelial growth factor (VEGF) is a potent mitogen and chemoattractant for endothelial cells and a key modulator of vasculogenesis and angiogenesis in physiological and pathological conditions (1). It also acts as a vascular permeability factor, increasing microvascular permeability (1).

VEGF is a dimeric glycoprotein synthesized and secreted by epithelial, mesenchymal and tumoral cells. Several isoforms, deriving from alternative splicing of the VEGF gene, have been identified in different organs and the three main splice variants are characterized by 121, 165 and 189 amino acids (VEGF 121, VEGF 165 and VEGF 189 respectively) (2, 3).

The physiological significance of the different VEGF isoforms is essentially unknown although experimental findings suggest that VEGF 121 and VEGF 165 isoforms may have distinctive activities at the renal level (4–7). The various VEGF isoforms bind to two tyrosine kinase receptors expressed by endothelial cells: VEGF receptor 1 (VEGF-R1; Flt-1), which seems important in regulating endothelial cell–cell and cell–matrix interactions (8, 9) and VEGF receptor 2 (VEGF-R2; KDR) which mediates the mitogenic and chemoattractant actions of VEGF (10–12).

The three main splice variants of VEGF are constitutively expressed in normal kidneys, mostly in the glomeruli by podocytes, but also in the tubulo-interstitium by tubular cells and in medulla by collecting duct

cells (13–15). Recently, two additional isoforms have been identified in human glomeruli: VEGF 145 and VEGF 148 (16). In the normal kidney, VEGF 165 is expressed at the same or at a higher level than the VEGF 121 isoform (14, 15), both VEGF-R2 and VEGF-R1 are expressed in glomerular endothelial cells (17) and VEGF-R1 constitutes 60% of VEGF receptors (18). Moreover, a soluble form of VEGF-R1 receptor (sFlt-1) has been demonstrated in normal human glomeruli (16, 19).

During fetal life VEGF plays a pivotal role in glomerulogenesis and tubulogenesis (20, 21) but the function of VEGF within the adult kidney is unknown. It has been hypothesized that VEGF expression within podocytes influences glomerular endothelial structure, function and permeability (17, 18).

The expression of VEGF in the kidney has been studied recently (22) but its role in renal physiology and pathology is still unclear. There is evidence that glomerular VEGF deficiency or VEGF 165 overexpression causes glomerular lesions such as those observed in acquired renal diseases in the 2.5-week-old mouse (22). Recent findings suggest that, in the kidney, VEGF exerts an action on endothelial integrity that differs from its mitogenic and angiogenic effects. Finally, VEGF has been defined as a survival factor, acting as an anti-apoptotic agent for endothelial cells (23, 24). As a consequence, some authors have speculated that, in normal conditions, VEGF secretion by podocytes and its binding to VEGF receptors on glomerular endothelial cells may act as a paracrine regulatory mechanism, necessary for the integrity and normal function of the glomerular endothelium (13, 14, 17, 22). This hypothesis suggests that, in glomerular diseases, alterations in VEGF expression may exist, either as a rescue or reparative mechanism or as a pathological consequence. Because of the activity of VEGF, these alterations might lead to impairment of glomerular function and permeability. In particular, VEGF actions on endothelium and capillary permeability and its vasoactive effects suggest that it could play a critical role in diabetic nephropathy (17, 18), as observed in diabetic retinopathy (25–28). However, evidence implicating VEGF in diabetic glomerulopathy is poor, and the precise role of VEGF in human diabetic nephropathy is still undetermined (25, 29).

To date, only a few studies have explored the expression of VEGF in the glomeruli of diabetic patients with nephropathy: VEGF, both at mRNA and protein level, has been demonstrated to be decreased in sclerotic areas and in Kimmelstiel–Wilson nodules in patients with advanced stages of disease (30–32). More recently, it has been shown that VEGF protein was increased in the glomeruli of patients with early diabetic nephropathy (33). These studies, however, do not provide quantitative data on VEGF gene expression or the expression pattern of the VEGF isoforms. Moreover, quantitative data on the degree of renal lesions

have not been reported; thus, the relationship between intraglomerular VEGF expression and the degree of glomerular injury remains unknown.

The aims of this study were to evaluate glomerular expression of VEGF and VEGF receptors and to study the relationships between VEGF gene expression and renal functional and structural parameters in type 2 diabetic patients. To this end, VEGF, VEGF-R1 and VEGF-R2 mRNAs were quantified and the expression pattern of VEGF isoforms was analysed in microdissected glomeruli of type 2 diabetic patients with various degrees of diabetic nephropathy.

Materials and methods

Seventeen type 2 diabetic patients were studied. Inclusion criteria were: (i) age less than 75 years; (ii) age at diagnosis of type 2 diabetes more than 40 years; (iii) known duration of diabetes of at least 2 years; (iv) serum creatinine less than 220 $\mu\text{mol/l}$; (v) absence of other renal diseases, as defined by clinical and biochemical parameters and by light, electron and immunofluorescence microscopic studies; (vi) no secondary forms of hypertension; and (vii) micro-albumin excretion rate (AER) 20–200 $\mu\text{g/min}$ or macroalbuminuria (AER > 200 $\mu\text{g/min}$).

After admission, patients underwent at least three 24 h urine collections for measurement of AER by immunoturbidimetric assay. Glomerular filtration rate (GFR) was determined by plasma clearance of ^{51}Cr -EDTA (34). Glycated hemoglobin (HbA1c) was measured by high-performance liquid chromatography to assess metabolic control. The clinical data of the type 2 diabetic patients are shown in Table 1. Ten patients were treated with insulin, four with oral agents and insulin and three with oral agents. All patients received angiotensin-converting enzyme (ACE) inhibitors alone (four patients) or associated with other anti-hypertensive agents. Patients underwent percutaneous renal biopsy under ultrasound guidance. Tissue was immediately observed under a dissecting microscope to ensure the presence of an adequate number of glomeruli, and processed for light microscopy and immunofluorescence. Of the 17 kidney biopsies, 14 were available for electron microscopic morphometric analysis (35) to estimate: mesangial fractional volume ($V_v(\text{mes}/\text{glom})$), i.e. the proportion of the glomerulus occupied by mesangium; mesangial cell ($V_v(\text{MC}/\text{glom})$) and mesangial matrix ($V_v(\text{MM}/\text{glom})$) fractional volumes; and glomerular basement membrane (GBM) width (35). Twenty-seven normal kidney donors (age 56 ± 10 years, 14 males and 13 females) at the University of Minnesota served as normal controls for the morphometric parameters.

These studies were approved by the ethical committee of the University of Padua and patients gave written informed consent.

Table 1 Clinical data of type 2 diabetic patients.

Patient no.	Gender	Age (years)	Diabetes duration (years)	HbA1C (%)	AER ($\mu\text{g}/\text{min}$)	GFR ($\text{ml}/\text{min}/1.73\text{ m}^2$)	Mean blood pressure (mmHg)
1	M	59	22	8.5	34	76.5	113.0
2	M	55	13	10.8	54.4	140	106.6
3	F	57	3	9.2	47.7	96.4	96.6
4	F	63	17	7.6	50.7	116	110.0
5	M	52	5	11.1	21.1	149	96.6
6	F	53	7	8.9	27	159	103.3
7	M	52	14	10.5	37.7	98.5	106.6
8	F	54	15	8.2	464	46	112.0
9	M	52	3	7.9	270	72	96.7
10	M	73	12	8.6	3880	na	104.0
11	M	74	7	7.9	3472	na	107.3
12	F	55	16	9.2	6944	na	108.0
13	M	59	30	8.3	154	87	103.3
14	M	59	13	8.1	3394	35	90.1
15	M	54	14	8.5	109	57	110.0
16	M	61	24	8.5	22.9	86	100.3
17	M	66	21	8.2	762	102	97.0

na = not available.

Microdissection and RNA extraction

Immediately after biopsy, approximately one-tenth of the specimen was kept in physiological solution containing 100 U RNase inhibitor (RNasin) (Applied Biosystems, Foster City, CA, USA) in ice. Four to fifteen glomeruli were isolated under a stereomicroscope (Zeiss, Jena, Germany) and immediately put into RNazolB solution (Biotex, Houston, TX, USA). Total RNA was extracted using the RNazol B method with some minor modifications as previously described (36).

Reverse transcription (RT)

Total RNA (30 ng) was reverse transcribed in a final volume of 20 μl , in the presence of 5 mmol/l MgCl_2 , 1 mM dNTP, 1 U/ μl RNase inhibitor, 2.5 U/ μl MuLV reverse transcriptase (Applied Biosystems) and 2.5 $\mu\text{mol}/\text{l}$ random examers (Applied Biosystems) in buffer (50 mmol/l KCl and 10 mM Tris-HCl, pH 8.3).

RT reaction was carried out for 30 min at 42 °C and 5 min at 99 °C in a thermocycler (MJ Research, Watertown, MA, USA).

Polymerase chain reaction (PCR)

A comparative kinetic RT-PCR approach was used to quantify VEGF, VEGF-R1 and VEGF-R2 mRNAs in each specimen (36). An aliquot of 1 μl RT sample was used to amplify, in different tubes, VEGF, VEGF-R1 and VEGF-R2 genes and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (G3PDH; Clontech, Palo Alto, CA, USA) as internal standard. The specific cDNA sequences were amplified using the primers reported in Table 2. Primers mapping in the region of the VEGF gene common to all isoforms were used to quantify total VEGF mRNA (Fig. 1). The amplification was carried out in a final volume of 25 μl containing: MgCl_2 (1.5 mmol/l for VEGF and G3PDH,

Table 2 Oligonucleotide primers used to amplify specific mRNAs.

Gene	Sequence (5'–3')	Product size (bp)
G3PDH	F: TGA AGG TCG GAG TCA ACG GAT TTG GT R: CAT GTG GGC CAT GAG GTC CAC CAC	983
G3PDH	F: ACC ACA GTC CAT GCC ATC AC R: TCC ACC ACC CTG TTG CTG TA	452
VEGF (quantitative analysis) (reference 36)	F: TCC ATG GAT GTC TAT CAG CG R: GCT CAT CTC TCC TAT GTG CT	234
VEGF (qualitative analysis) (reference 10)	F: ATG CGG ATC AAA CCT CAC C R: ATC TGG TTC CCG AAA CCC TG	363 (VEGF189) 291 (VEGF165) 159 (VEGF121)
VEGF-R2	F: GAC AAG ACA GCA ACT TGC R: GAT TGT GTA CAC TCT GTC A	684
VEGF-R1 (reference 37)	F: GAA GGC ATG AGG ATG AGA GC R: CAG GCT CAT GAA CTT GAA AGC	324

Figure 1 Gene structure of the different VEGF splice variants expressed in the kidney. The solid symbols mark the position of sense and antisense primers for mRNA quantitative analysis (◆) and for the detection of each isoform (●).

2.5 mmol/l for VEGF-R2 and 2 mmol/l for VEGF-R1), 0.2 mmol/l dNTP, 2 U JumpStart Taq DNA polymerase (Sigma, St Louis, MO, USA), 0.4 μ mol/l primers in 50 mmol/l KCl and 10 mmol/l Tris-HCl, pH 8. VEGF-R2 primers were used at 1.16 mmol/l. cDNA was amplified according to the following conditions: VEGF 94 °C for 45 s, 55 °C for 1 min and 72 °C for 2 min; G3PDH and VEGF-R1 94 °C for 45 s, 60 °C for 45 s and 72 °C for 2 min; VEGF-R2 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. RT-PCR analysis revealed that in all samples the internal standard G3PDH was always amplified and a clear signal was visible at 35 cycles. Since the quantity of extracted RNA from glomeruli was very low, it was not possible to analyze RNA integrity by ethidium bromide gel. To this purpose, we applied a previously validated indirect method (36), i.e. the amplification of 986 bp G3PDH. The amplifiability of G3PDH ensured that the RNAs in the samples were not degraded and that the efficiency of the RT reaction was comparable between samples (36). Thus, after determining for each gene in each sample the exponential phase of reaction by kinetic PCR (data not shown), we selected the best cycles in which PCR products should be quantitated: 35 cycles for G3PDH, 31 cycles for VEGF, 33 cycles for VEGF-R2 and 34 cycles for VEGF-R1 (Fig. 2).

To confirm the validity of our semiquantitative comparative RT-PCR approach, the real-time PCR assay on the light cycler system described by Wellman *et al.* (37) was used to quantify total VEGF mRNA in six type 2 diabetic patients in which sufficient RNA was available. Regression analysis was used to compare total VEGF mRNA levels obtained by kinetic comparative RT-PCR

Figure 2 VEGF and VEGF receptor RT-PCR products generated by (A) 31, (B) 33 and (C) 34 amplification cycles.

and by real-time PCR; a significant correlation ($r = 0.92$, $P < 0.05$) was observed, confirming that our comparative kinetic RT-PCR approach gives reliable quantitative information.

The different VEGF isoforms were analysed by kinetic RT-PCR using primers spanning the splice sites of VEGF gene (Fig. 1 and Table 2). According to Nicol *et al.* (15) these primers generate three amplification products of 159, 291 and 363 bp corresponding to VEGF 121, VEGF 165 and VEGF 189 respectively (Fig. 3). Control negative reactions, without reverse transcriptase, were performed during the cDNA synthesis step in order to exclude genomic contamination.

In a final volume of 25 μ l containing 1.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTP, 2 U JumpStart Taq DNA polymerase and 0.4 μ mol/l primers in 50 mmol/l KCl and 10 mmol/l Tris-HCl, pH 8, cDNA was amplified according to the following conditions: 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min. From 34 to 44 amplification cycles, 5 μ l PCR was taken for subsequent PAGE analysis every two cycles.

PAGE and densitometric analysis

RT-PCR products were analyzed by PAGE followed by silver staining as previously described (36). Gel bands were quantified directly in the gel by densitometric

Figure 3 PAGE analysis of VEGF isoforms relative to patient 10. RT-PCR products were generated by sequential amplification cycles from 34 to 44.

analysis using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA). Quantitative analysis of VEGF, VEGF-R1 and VEGF-R2 expression was performed by comparing the ratio of PCR product optical density (OD) generated from target and standard templates (OD target/OD G3PDH), corrected for differences in the molecular weight of the amplicons.

Quantification of VEGF isoforms was provided by the semiquantitative RT-PCR method in which the internal control was the other co-amplified VEGF isoform; thus we determined in each sample the comparative expression of each VEGF isoform as a percentage calculated from the total sum of each isoform OD (38, 39).

Statistical analysis

Data are expressed as means $\pm$ S.D. Values for AER, not normally distributed, were logarithmically transformed before analysis and are expressed as median and range. Correlations between variables were assessed using linear regression analysis. Comparison between groups was performed by two-tailed unpaired *t*-test. Statistical significance level was defined at $P < 0.05$.

Results

The results of morphometric analysis on glomerular structure are shown in Table 3: Vv(mes/glom), Vv(MM/glom) and GBM width were significantly increased in diabetic patients compared with controls: Vv(mes/glom) 0.27 ± 0.07 vs 0.19 ± 0.03 ($P < 0.01$), Vv(MC/glom) 0.07 ± 0.02 vs 0.09 ± 0.03 (not significant), Vv(MM/glom) 0.16 ± 0.06 vs 0.09 ± 0.02

($P < 0.01$) and GBM width 471 ± 81 vs 310 ± 38 nm ($P < 0.01$).

Vv(mes/glom) was directly related to AER ($r = 0.71$, $P = 0.0043$) and inversely to GFR ($r = -0.73$, $P = 0.003$); no correlations between other structural and functional parameters were found.

Total VEGF and its isoforms

PAGE analysis of RT-PCR products in microdissected glomeruli showed the signal corresponding to total VEGF mRNA, as the expected band of 234 bp (Fig. 2A). The signal was detectable in all samples; in three patients, in whom the band did not allow densitometric analysis, 0.00 was conventionally assigned (patient numbers 10, 12 and 14 in Table 3). To obtain data on VEGF isoform expression, we used kinetic RT-PCR, sampling PCR products from 34 to 44 cycles (Fig. 3). PAGE analysis showed that, at every cycle that was considered, the predominant isoform was VEGF 121, while VEGF 189 was only barely detectable. The distribution of the different isoforms among patients is reported in Table 3, where the level of expression of each isoform is given as the mean of percentages calculated at every amplification cycle. A strong inverse correlation between VEGF 165 and VEGF 121 isoforms ($r = -0.86$, $P = 0.0001$) was found. To verify whether the observed inverse relationship of increased shorter and decreased longer VEGF isoform PCR product could not be the result of preferential amplification of shorter amplicons, experiments using different amounts of initial cDNA (30, 100 and 200 ng) were performed. We established that the VEGF isoform pattern was independent of the starting

Table 3 Relative levels of VEGF and VEGF receptor mRNAs in microdissected glomeruli and morphometric parameters. Isoform percentage is the mean of values obtained at every amplification cycle ($\pm$ S.D.).

Patient no.	VEGF	VEGF 189 (%)	VEGF 165 (%)	VEGF 121 (%)	VEGF-R2	VEGF-R1	Vv(mes/glom)	Vv(MM/glom)	Vv(MC/glom)	GBM (nm)
1	1.66	1.3 ± 0.7	19.8 ± 10.6	78.9 ± 6.9	0.20	0.28	0.29	0.17	0.07	532
2	1.12	4.8 ± 2.6	31.2 ± 11.9	64.0 ± 7.0	0.24	0.36	0.24	0.1	0.08	383
3	1.00	5.6 ± 3.9	36.0 ± 4.0	58.4 ± 7.3	0.66	0.75	0.22	0.11	0.08	397
4	0.69	4.3 ± 2.7	40.0 ± 2.6	55.7 ± 5.2	0.33	0.47	0.21	0.13	0.05	480
5	1.07	3.0 ± 3.3	41.4 ± 3.4	55.6 ± 6.6	0.29	0.27	0.23	0.13	0.06	534
6	0.34	2.1 ± 1.3	17.8 ± 9.4	80.1 ± 10.2	0.00	0.23	0.2	0.15	0.03	361
7	0.65	1.2 ± 1.2	25.0 ± 5.6	73.8 ± 6.7	0.76	0.74	0.28	0.15	0.07	590
8	0.39	0.6 ± 0.8	12.8 ± 6.4	86.6 ± 7.5	0.00	0.44	0.43	0.31	0.10	515
9	0.21	4.9 ± 5.7	37.2 ± 4.9	57.9 ± 9.5	0.00	0.00	0.22	0.14	0.04	389
10	0.00*	0.0 ± 0.0	11.1 ± 9.3	88.9 ± 9.3	0.00	0.00	na	na	na	na
11	0.34	0.0 ± 0.0	24.0 ± 2.8	76.0 ± 2.8	0.02	0.29	na	na	na	na
12	0.00*	0.0 ± 0.0	0.0 ± 0.0	100 ± 0.0	0.18	0.16	na	na	na	na
13	0.04	0.0 ± 2.6	33.0 ± 12.2	67.0 ± 14.0	0.00	0.05	0.33	0.16	0.10	529
14	0.00*	0.7 ± 2.4	5.6 ± 4.1	93.7 ± 6.59	0.00	0.00	0.55	0.29	0.17	867
15	0.12	1.4 ± 2.6	18.4 ± 7.9	80.2 ± 10.2	0.07	0.11	0.29	0.19	0.03	494
16	0.13	2.2 ± 1.46	34.8 ± 3.4	63.0 ± 4.79	0.14	0.85	0.26	0.18	0.02	520
17	0.31	1.5 ± 2.17	28.1 ± 4.53	70.4 ± 6.35	0.19	1.77	0.25	0.17	0.02	472

na = not available.

*Values conventionally assigned to VEGF signals not evaluable by densitometric analysis.

Figure 4 Correlation between AER and VEGF mRNA levels ($r = -0.66$, $P = 0.004$).

quantity of RNA (data not shown) and of the number of amplification cycles.

There was a significant inverse correlation between total intraglomerular VEGF mRNA level and AER ($r = -0.66$, $P = 0.004$) (Fig. 4); no other significant correlation was found between total VEGF mRNA levels and other functional or structural parameters.

Vv(mes/glom) and Vv(MM/glom) were inversely correlated with the percentage of VEGF 165 ($r = -0.73$, $P = 0.005$ and $r = -0.64$, $P = 0.017$ respectively), and directly with the percentage of VEGF 121 ($r = 0.74$, $P = 0.003$ and $r = 0.73$, $P = 0.004$ respectively) (Fig. 5).

VEGF receptors (VEGF-R1 and VEGF-R2)

PAGE analysis showed PCR products of the expected size (Fig. 2B and C, and Table 2). Densitometric analysis revealed that mRNA levels were highly variable (range 0–0.76 and 0–1.77 for VEGF-R2 and VEGF-R1 respectively; Table 3).

VEGF and VEGF-R2 mRNA levels were directly related ($r = 0.62$, $P = 0.033$). In contrast there was no correlation between VEGF and VEGF-R1 and between VEGF-R1 and VEGF-R2 mRNAs. Moreover VEGF-R1 and VEGF-R2 mRNA levels were not related to any functional and structural parameters.

Discussion

The results of the present study suggest that quantitative and qualitative changes in glomerular VEGF expression are present in type 2 diabetic patients with diabetic nephropathy and are related to the degree of albuminuria and mesangial expansion.

In streptozotocin-diabetic rats, conflicting results have been reported; while Gudehithlu *et al.* (40) demonstrated that in early diabetes glomerular VEGF protein is decreased, other authors have described an increase in VEGF mRNA and protein in glomeruli in short-term diabetic rats (4, 41). In the *db/db* mouse, a model of obese type 2 diabetes, Flyvberg *et al.* (42) have provided indirect evidence on the relevance of VEGF in renal complication. In fact, although data on VEGF expression were not provided, they demonstrated that chronic inhibition of VEGF by anti-VEGF antibodies was associated with an amelioration in albuminuria and diabetic glomerular lesions. In glomeruli of patients in the early phase of diabetic kidney disease, Cha *et al.* (33) reported increased levels of VEGF protein.

The results of the present study indicate that in type 2 diabetic patients the glomerular content of VEGF mRNA decreases as the AER increases. It is probably true that, as suggested by Flyvberg *et al.* (42), VEGF acts by increasing capillary permeability, and it is possible that up-regulation of VEGF contributes to the early

Figure 5 Correlation between VEGF 121 and VEGF 165 isoform expression and morphometric parameters.

changes of diabetic nephropathy and then, with the worsening of diabetic nephropathy, VEGF expression decreases.

Indeed, in glomeruli of diabetic patients with advanced nephropathy, immunohistochemistry and *in situ* hybridization techniques demonstrated that VEGF expression was decreased in sclerotic areas and in mesangial nodules (32, 33). Our results are in keeping with these data; in fact the four patients with heavy proteinuria and severe glomerulosclerosis had the lowest levels of VEGF. This is not surprising since, in the advanced stages of diabetic glomerulopathy, the majority of the tuft is occupied by extracellular matrix with a reduction in the number of cells. It is important to remember that the glomerular filtration barrier is a complex structure composed of fenestrated endothelium, GBM and by podocyte foot processes and slit diaphragms, which represent the main size-selective filter barrier in the kidney. In the normal kidney (13, 14) and in early diabetic nephropathy (41) most VEGF is produced by podocytes; in advanced diabetic nephropathy, where a reduction in the number of podocytes has been documented (43), it can be expected that glomerular VEGF expression is reduced. The findings in previous studies that urinary VEGF excretion increases with the progression of proteinuria may be related to an increase in the production of VEGF by tubular epithelial cells. Another important factor to take into consideration is diabetes duration. Indeed Cooper *et al.* (41) described a marked increase in gene expression of VEGF and VEGF-R2 in the kidneys of short-term diabetic rats, while the VEGF expression was only moderately increased in long-term diabetic rats, where there was no increase in VEGF-R2 expression.

We are aware that ACE inhibition might reduce VEGF expression (44) and all our patients were treated with ACE inhibitors; however, virtually all patients with type 2 diabetes are hypertensive and it might be dangerous to stop anti-hypertensive therapy. In addition, uncontrolled hypertension may induce mechanical stretch at the glomerular level and increase VEGF expression (45).

The major finding of our study is that matrix mesangial expansion is associated with a decrease of VEGF 165 mRNA levels with a proportional increase of VEGF 121. Dynamic VEGF isoform shift has been reported in pathological and physiological conditions (46, 47). It has been reported that, in the normal human kidney, VEGF 165 and VEGF 121 are equally abundant and that their ratio does not change either in glomeruli or total cortex (14). Indeed, in the diabetic patients with almost normal glomerular structure (patient numbers 4, 5 and 9) comparable levels of the two different isoforms were detected. The strong inverse correlation between VEGF 121 and VEGF 165 mRNA levels and the relationship between each isoform and the severity of mesangial matrix expansion,

the crucial lesion of diabetic nephropathy, prompted us to speculate that this pattern of VEGF isoform expression may be relevant to the pathogenesis of diabetic glomerulopathy. Interestingly, data have recently been reported on the effect of hyperglycemia on VEGF and the pathological consequences of this phenomenon (48). Actually, it has been shown that high glucose hampers epithelial mesenchymal transformation of endocardial cells by reducing myocardial VEGF-A and that this effect is reversed by exogenous VEGF 165 (48) suggesting that diabetes induces a specific VEGF 165 deficiency.

VEGF isoforms differ in their binding affinity to extracellular matrix, the 165 isoform being more avidly bound by extracellular matrix than the 121 one (1). Furthermore, VEGF 165 is a more potent functional ligand of VEGF-R2, and seems to play a major role in adult life (12) compared with VEGF 121 (49). Thus, the two isoforms could have different abilities to reach glomerular endothelial cells and to regulate their function. Recent studies have demonstrated that the VEGF 165 isoform could contribute to the preservation of normal glomerular structure (50). Sugimoto *et al.* (51) have recently demonstrated that, in normal mice, anti-VEGF antibodies cause rapid cell detachment and hypertrophy and proteinuria; these effects were inhibited by the simultaneous administration of human VEGF 165. In the anti-Thy 1.1 experimental model of mesangioproliferative nephritis, Ostendorf *et al.* (5), demonstrated that blocking VEGF 165 with the administration of an aptamer-based antagonist of VEGF 165 leads to an increase in the frequency of glomerular microaneurysms and proteinuria. Furthermore Masuda *et al.* (6), in a different model of mesangiolytic glomerulonephritis, demonstrated that the intraperitoneal injection of VEGF 165 enhances glomerular capillary repair and accelerates resolution. It is interesting to note that in this experimental model the main lesion is mesangiolysis, which leads to the formation of glomerular capillary microaneurysms. Similar phenomena occur in diabetic glomerulopathy and seem to be responsible for Kimmelstiel–Wilson nodules. Thus, we hypothesize that, given the reparative role of VEGF 165 (5, 6), a relative reduction of VEGF 165 may play a causative role in the development of diabetic glomerulopathy. On the other hand, mice expressing only the VEGF 121 isoform develop glomerulosclerosis by 6 weeks of age (52).

We observed a direct correlation between VEGF-R2 and VEGF expression; in view of the role of the VEGF-R2 on endothelial cell mitogenesis (10, 11) and on angiogenesis in the adult (12), this finding suggests the possible existence of a cross-talk, and prompted us to advance the hypothesis that VEGF plays a role in the remodeling of the glomerulus in diabetic nephropathy. VEGF-R1 are important in cell–cell and cell–matrix interactions (8, 9) and thus may be important regulators of capillary permeability.

Lack of a relationship between expression levels of VEGF and VEGF-R1 suggests that VEGF has no major glomerular capillary permeability effect. The finding that in micro- and macro-albuminuric diabetic patients there is no correlation between AER and VEGF-R1 supports this idea.

In conclusion, this study has demonstrated that quantitative and qualitative changes in glomerular VEGF expression are present in type 2 diabetic patients with diabetic nephropathy. These findings, along with recent reports (39, 50, 52, 53), suggest that VEGF 165 is important in maintaining the integrity of glomerular structure and that the isoform switch and/or the lowering of the level of VEGF 165 may be involved in the abnormal remodeling of the capillary tuft, extracellular matrix accumulation and thus in the pathogenesis of human diabetic glomerulopathy.

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References

- Ferrara N. Role of vascular endothelial growth factor in the regulation of angiogenesis. *Kidney International* 1999 **56** 794–814.
- Kretzler M, Schröppel B, Merkle M, Huber S, Mundel P, Horster M & Schlondorff D. Detection of multiple vascular endothelial growth factor splice isoforms in single glomerular podocytes. *Kidney International* 1998 **54** (Suppl 67) 159–161.
- Ferrara N & Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocrine Reviews* 1997 **18** 4–25.
- Tsuchida K, Makita Z, Yamagishi S, Atsumi T, Miyoshi H, Obara S, Ishida M, Ishikawa S, Yasumura K & Koike T. Suppression of transforming growth factor beta and vascular endothelial growth factor in diabetic nephropathy in rats by a novel advanced glycation end product inhibitor, OPB-9195. *Diabetologia* 1999 **42** 579–588.
- Ostendorf T, Kunter U, Eitner F, Loos A, Regele H, Kerjaschki D, Henninger DD, Janjic N & Floege J. VEGF 165 mediates glomerular endothelial repair. *Journal of Clinical Investigation* 1999 **104** 913–923.
- Masuda Y, Shimizu A, Mori T, Ishiwata T, Kitamura H, Ohashi R, Ishizaki M, Asano G, Sugisaki Y & Yamanaka N. Vascular endothelial growth factor enhances glomerular capillary repair and accelerates resolution of experimentally induced glomerulonephritis. *American Journal of Pathology* 2001 **159** 599–608.
- Kim YG, Suga SI, Kang DH, Jefferson JA, Mazzali M, Gordon KL, Matsui K, Breiteneder-Geleff S, Shankland SJ, Hughes J, Kerjaschki D, Schreiner GF & Johnson RJ. Vascular endothelial growth factor accelerates renal recovery in experimental thrombotic microangiopathy. *Kidney International* 2000 **58** 2390–2399.
- Fong GH, Rossant J, Gertsenstein M & Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 1995 **376** 66–70.
- Clauss M, Weich H, Breier G, Knies U, Rocki W, Waltenberger J & Risau W. The vascular endothelial growth factor receptor Flt-1 mediates biological activities. *Journal of Biological Chemistry* 1996 **271** 17629–17634.
- Millauer B, Witzmann-Voos S, Schnurch H, Martinez R, Moller NP, Risau W & Ullrich A. High affinity binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 1993 **72** 835–846.
- Waltenberger J, Claesson-Wells L, Siegbahn A, Shibuya M & Helding CH. Different signal transduction properties of KDR and Flt-1 two receptors for vascular endothelial growth factor. *Journal of Biological Chemistry* 1994 **269** 26988–26995.
- Ortega N, Jonca F, Vincent S, Favard C, Ruchoux MM & Plouet J. Systemic activation of the vascular endothelial growth factor receptor KDR/flk-1 selectively triggers endothelial cells with an angiogenic phenotype. *American Journal of Pathology* 1997 **151** 1215–1224.
- Brown LE, Berse B, Tognazzi K, Manseau EJ, Van De Water L, Senger DR, Dvorak HF & Rosen S. Vascular permeability factor mRNA and protein expression in human kidney. *Kidney International* 1992 **42** 1457–1461.
- Simon M, Gröne HJ, Jöhren O, Kullmer J, Plate KH, Risau W & Fuchs E. Expression of vascular endothelial growth factor and its receptors in human renal ontogenesis and in adult kidney. *American Journal of Physiology* 1995 **268** 240–250.
- Nicol D, Hii S, Walsh M, Teh B, Thompson L, Kennett C & Gotley D. Vascular endothelial growth factor expression is increased in renal cell carcinoma. *Journal of Urology* 1997 **157** 1482–1486.
- Whittle C, Gillespie K, Harrison R, Mathieson PW & Harper SJ. Heterogeneous vascular endothelial growth factor (VEGF) isoform mRNA and receptor mRNA expression in human glomeruli, and the identification of VEGF₁₄₈ mRNA, a novel truncated splice variant. *Clinical Science* 1999 **97** 303–312.
- Thomas S, Vanuystel J, Gruden G, Rodriguez V, Burt D, Gnudi L, Hartley B & Viberti G. Vascular endothelial growth factor receptors in human mesangium *in vitro* and in glomerular disease. *Journal of the American Society of Nephrology* 2000 **11** 1236–1243.
- Simon M, Röckl W, Hornig C, Grone EF, Theis H, Weich HA, Fuchs E, Yayon A & Grone HJ. Receptors of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) in fetal and adult human kidney localization and [¹²⁵I]VEGF binding sites. *Journal of the American Society of Nephrology* 1998 **9** 1032–1044.
- Williams B. A potential role for angiotensin II induced vascular endothelial growth factor expression in the pathogenesis of diabetic nephropathy? *Mineral and Electrolyte Metabolism* 1998 **24** 400–405.
- Kitamoto Y, Tokunaga H & Tomita K. Vascular endothelial growth factor is an essential molecule for mouse kidney development of glomerulogenesis and nephrogenesis. *Journal of Clinical Investigation* 1997 **99** 2351–2357.
- Tufro A, Norwood VF, Carey RM & Gomez RA. Vascular endothelial growth factor induces nephrogenesis and vasculogenesis. *Journal of the American Society of Nephrology* 1999 **10** 2125–2134.
- Eremina V, Sood M, Haigh J, Nagy A, Lajoie G, Ferrara N, Gerber HP, Kikkawa Y, Miner JH & Quaggin SE. Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal disease. *Journal of Clinical Investigation* 2003 **111** 707–716.
- Gerber HP, Dixit V & Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *Journal of Biological Chemistry* 1998 **273** 13313–13316.
- Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V & Ferrara N. VEGF regulates endothelial cell survival by the PI3-kinase/Akt signal transduction pathway requirement for

- Flk-1/KDR activation. *Journal of Biological Chemistry* 1998 **273** 30336–30343.
- 25 Duh E & Aiello LP. Vascular endothelial growth factor and diabetes. The agonist versus antagonist paradox. *Diabetes* 1999 **48** 1899–1906.
 - 26 Paques M, Massin P & Gaudric A. Growth factors and diabetic retinopathy. *Diabetes and Metabolism* 1997 **23** 125–130.
 - 27 Aiello LP & Wong JS. Role of vascular endothelial growth factor in diabetic vascular complications. *Kidney International* 2000 **58** (Suppl 77) S113–S119.
 - 28 Tilton RG, Kawamura T, Chang KC, Ido Y, Bjercke B, Stephan CC, Brock TA & Williamson JR. Vascular dysfunction induced by elevated glucose levels in rats is mediated by vascular endothelial growth factor. *Journal of Clinical Investigation* 1997 **99** 2192–2202.
 - 29 Del Prete D, Anglani F, Ceol M, D'Angelo A, Forino M, Vianello D, Baggio B & Gambaro G. Molecular biology of diabetic glomerulosclerosis. *Nephrology, Dialysis, Transplantation* 1998 **13** (Suppl 8) 20–25.
 - 30 Gröne HJ, Simon M & Gröne E. Expression of vascular endothelial growth factor in renal vascular disease and renal allografts. *Journal of Pathology* 1995 **177** 259–267.
 - 31 Shulman K, Rosen S, Tagnazzi K, Manseau EJ & Brown LF. Expression of vascular permeability factor (VPF/VEGF) is altered in many glomerular diseases. *Journal of the American Society of Nephrology* 1996 **7** 661–666.
 - 32 Bailey E, Bottomley MJ, Westwell S, Pringle JH, Furness PN, Feehally J, Brenchley PE & Harper SJ. Vascular endothelial growth factor mRNA expression in minimal change, membranous, and diabetic nephropathy demonstrated by non-isotopic *in situ* hybridisation. *Journal of Clinical Pathology* 1999 **52** 735–738.
 - 33 Cha DR, Kim NH, Yoon JW, Jo SK, Cho WY, Kim HK & Won NH. Role of vascular endothelial growth factor in diabetic nephropathy. *Kidney International* 2000 **58** (Suppl 77) S104–S112.
 - 34 Sambataro M, Thomaseth K, Pacini G, Robaudo C, Carraro A, Bruseghin M, Brocco E, Abaterusso C, DeFerrari G, Fioretto P, Maioli M, Tonolo GC, Crepaldi G & Nosadini R. Plasma clearance of ⁵¹Cr-EDTA provides a precise and convenient technique for measurement of glomerular filtration rate in diabetic humans. *Journal of the American Society of Nephrology* 1996 **7** 1–10.
 - 35 Fioretto P, Steffes MW & Mauer M. Glomerular structure in non proteinuric IDDM patients with various levels of albuminuria. *Diabetes* 1994 **43** 1358–1364.
 - 36 Del Prete D, Forino M, Gambaro G, D'Angelo A, Baggio B & Anglani F. A comparative kinetic RT/PCR strategy for the quantitation of mRNAs in microdissected human renal biopsy specimens. *Experimental Nephrology* 1998 **6** 563–567.
 - 37 Wellman S, Taube T, Paal K, Graf V, Einsiedel H, Geilen W, Seifert G, Eckert C, Henze G & Seeger K. Specific reverse transcription-PCR quantification of vascular endothelial growth factor (VEGF) splice variants by light cycler technology. *Clinical Chemistry* 2001 **47** 654–660.
 - 38 Whittle C, Gillespie K, Harrison R, Mathieson P & Harper J. Heterogeneous vascular endothelial growth factor (VEGF) isoform mRNA and receptor mRNA expression in human glomeruli, and the identification of VEGF148 mRNA, a novel truncated splice variant. *Clinical Science* 1999 **97** 303–312.
 - 39 Latil A, Bièche I, Pesche S, Valéri A, Fournier G, Cussenot O & Lidereau R. VEGF overexpression in clinically localized prostate tumors and neuropilin-1 overexpression in metastatic forms. *International Journal of Cancer* 2000 **89** 167–171.
 - 40 Gudehithlu KP, Pegoraro AA, Wanna L, Arruda JAL, Dunea G & Singh AK. Matrix accumulation in diabetes an imbalance between pro- and anti-angiogenic factors. *Journal of the American Society of Nephrology* 1999 **10** (Abstract) 681.
 - 41 Cooper ME, Vranes D, Youssef S, Stacker SA, Cox AJ, Rizkalla B *et al.* Increased renal expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 in experimental diabetes. *Diabetes* 2000 **48** 2229–2239.
 - 42 Flyvbjerg A, Dagnaes-Hansen E, De Vriese AS, Schrijvers BF, Tilton RG & Rasch R. Amelioration of long-term renal changes in obese type 2 diabetic mice by a neutralizing vascular endothelial growth factor antibody. *Diabetes* 2002 **51** 3090–3094.
 - 43 Dalla Vestra M, Masiero A, Roiter AM, Saller A, Crepaldi G & Fioretto P. Is podocyte injury relevant in diabetic nephropathy? Studies in patients with type 2 diabetes. *Diabetes* 2003 **52** 1031–1035.
 - 44 Gilbert RE, Kelly DJ, Cox AJ, Wilkinson-Berka JL, Rumble JR, Osicka T, Panagiotopoulos S, Lee V, Hendrich EC, Jerums G & Cooper ME. Angiotensin converting enzyme inhibition reduces retinal overexpression of vascular endothelial growth factor and hyperpermeability in experimental diabetes. *Diabetologia* 2000 **43** 1360–1367.
 - 45 Gruden G, Thomas S, Burt D, Lane S, Chusney G, Sacks S & Viberti G. Mechanical stretch induces vascular permeability factor in human mesangial cells; mechanisms of signal transduction. *PNAS* 1997 **94** 12112–12116.
 - 46 De Boer RA, Henning RH, Tio RA, Pinto YM, Brouwer RM, Ploeg RJ, Bohm M, Van Gilst WH & Van Veldhuisen DJ. Identification of a specific pattern of downregulation in expression of isoforms of vascular endothelial growth factor in dilated cardiomyopathy. *Heart* 2002 **88** 412–414.
 - 47 Ancelin M, Buteau-Lozano H, Meduri G, Osborne-Pellegrin M, Sordello S, Plouet J & Perrot-Appanet M. A dynamic shift of VEGF isoforms with a transient and selective progesterone-induced expression of VEGF 189 regulates angiogenesis and vascular permeability in human uterus. *PNAS* 2002 **99** 6023–6028.
 - 48 Enciso JM, Gratzinger D, Camenisch TD, Canosa S, Pinter E & Madri JA. Elevated glucose inhibits VEGF-A-mediated endocardial cushion formation modulation by PECAM-1 and MMP-2. *Journal of Cell Biology* 2003 **160** 605–615.
 - 49 Whitaker GB, Limberg BJ & Rosenbaum JS. Vascular endothelial growth factor receptor-2 and neuropilin-1 form a receptor complex that is responsible for the differential signaling potency of VEGF (165) and VEGF (121). *Journal of Biological Chemistry* 2001 **276** 25520–25531.
 - 50 Ziyadeh FN & Wolf G. Why should an angiogenic factor modulate tubular structure in diabetic nephropathy? Some answers, more questions. *Kidney International* 2003 **64** 758–759.
 - 51 Sugimoto H, Hamano Y, Charytan D, Cosgrove D, Kieran M, Sudhakar A & Kalluri R. Neutralization of circulating vascular endothelial growth factor (VEGF) by anti-VEGF antibodies and soluble VEGF receptor 1 (sFlt-1) induces proteinuria. *Journal of Biological Chemistry* 2003 **278** 12605–12608.
 - 52 Mattot V, Moons L, Lupu F, Chernavsky D, Gomez RA, Collen D & Carmeliet P. Loss of the VEGF (164) and VEGF (188) isoforms impairs postnatal glomerular angiogenesis and renal arteriogenesis in mice. *Journal of the American Society of Nephrology* 2002 **13** 1548–1560.
 - 53 Foster RR, Hole R, Anderson K, Satchell SC, Coward RJ, Mathieson PW, Gillatt DA, Saleem MA, Bates DO & Harper SJ. Functional evidence that vascular endothelial growth factor may act as an autocrine factor on human podocytes. *American Journal of Physiology* 2003 **284** F1263–F1273.

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