Interstitial Vascular Rarefaction and Reduced VEGF-A Expression in Human Diabetic Nephropathy

Maja T. Lindenmeyer,* Matthias Kretzler,*† Anissa Boucherot,* Silvia Berra,‡ Yoshinari Yasuda,* Anna Henger,† Felix Eichinger,*† Stefanie Gaiser,* Holger Schmid,* Maria P. Rastaldi,‡ Robert W. Schrier,§ Detlef Schlöndorff,* and Clemens D. Cohen*

**Nephrologisches Zentrum, Medizinische Poliklinik, University of Munich, Munich, Germany; †Department of Medicine, University of Michigan, Ann Arbor, Michigan; ‡Renal Immunopathology Laboratory, Fondazione D'Amico per la Ricerca sulle Malattie Renali, c/o San Carlo Borromeo Hospital, Milan, Italy; and §Department of Medicine, University of Colorado, Denver, Colorado*

Diabetic nephropathy (DN) is a frequent complication in patients with diabetes. Although the majority of DN models and human studies have focused on glomeruli, tubulointerstitial damage is a major feature of DN and an important predictor of renal dysfunction. This study sought to investigate molecular markers of pathogenic pathways in the renal interstitium of patients with DN. Microdissected tubulointerstitial compartments from biopsies with established DN and control kidneys were subjected to expression profiling. Analysis of candidate genes, potentially involved in DN on the basis of common hypotheses, identified 49 genes with significantly altered expression levels in established DN in comparison with controls. In contrast to some rodent models, the growth factors vascular endothelial growth factor A (VEGF-A) and epidermal growth factor (EGF) showed a decrease in mRNA expression in DN. This was validated on an independent cohort of patients with DN by real-time reverse transcriptase–PCR. Immunohistochemical staining for VEGF-A and EGF also showed a reduced expression in DN. The decrease of renal VEGF-A expression was associated with a reduction in peritubular capillary densities shown by platelet-endothelial cell adhesion molecule-1/CD31 staining. Furthermore, a significant inverse correlation between VEGF-A and proteinuria, as well as EGF and proteinuria, and a positive correlation between VEGF-A and hypoxia-inducible factor-1α mRNA was found. Thus, in human DN, a decrease of VEGF-A, rather than the reported increase as described in some rodent models, may contribute to the progressive disease. These findings and the questions about rodent models in DN raise a note of caution regarding the proposal to inhibit VEGF-A to prevent progression of DN.

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The industrialized world faces an epidemic of obesity and type 2 diabetes with significant consequences for health systems and challenges for most medical areas of expertise, not the least of which nephrology. Diabetic nephropathy (DN) has become the most frequent cause of ESRD in developed countries (1). A common feature of early-stage DN is the development of albuminuria, which is associated with glomerular hypertrophy, thickening of glomerular basement membrane, and expansion of mesangial extracellular matrix. Advanced DN is characterized by glomerulosclerosis, vascular and capillary rarefaction, tubulointerstitial degeneration, and fibrosis that is associated with decline of GFR and substantial proteinuria (2–4). It is widely accepted that tubulointerstitial damage correlates with the degree of renal dysfunction and that it is a reliable prognosis factor for ESRD (3,5,6).

Vascular endothelial growth factor A (VEGF-A) is an important endothelial cell angiogenic, survival, and trophic factor. It is constitutively expressed in human and rodent kidneys, with podocytes representing a prominent source of this factor and epithelial cells of the tubulus and collecting duct as additional producers (7–9). The glomerular and peritubular capillary network is critical for maintenance of kidney function (10,11). Studies of rodent models of progressive renal failure have shown that the progressive loss in glomerular and tubular VEGF-A coincides with loss of glomerular and peritubular capillaries and the development of glomerulosclerosis and interstitial fibrosis (11). In rodent models of DN, studies indicate that renal (9,12,13) as well as glomerular (9,13–15) VEGF-A levels are enhanced. An increased expression of VEGF-A in the glomerular, tubulointerstitial, and vascular compartments was shown in streptozotocin-induced diabetic rats (9,12). Increased renal and/or glomerular VEGF-A production has been also described in experimental models of type 2 diabetes (13–15). The reports of increased VEGF-A expression in rodent models as well as in human diabetic retinopathy (16,17) led to the hypothesis that neoangiogenesis induced by VEGF-A may contribute to the development of DN and, therefore, that blocking VEGF-A or its signaling may prevent or ameliorate DN (16,18–20). Recent studies using rodent models of DN seem to support

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Address correspondence to: Dr. Clemens D. Cohen, Nephrologisches Zentrum, Medizinische Poliklinik, University of Munich, Pettenkoferstrasse 8a, 80336 Munich, Germany. Phone: +49-89-218075845; Fax: +49-89-218075860; E-mail: clemens.cohen@med.uni-muenchen.de

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this hypothesis (21,22), whereas others failed to demonstrate an effect of VEGF inhibition on disease progression (23). In humans, inhibition of VEGF-A as tumor therapy can lead to proteinuria as an adverse effect (24). The interpretation of rodent studies has to take into account the nonprogressive nature of the current murine DN models and their limited applicability to progressive human DN (25). Data regarding VEGF-A expression in patients with diabetes have to date focused on the glomerulus and have provided inconsistent results (26–28). Using immunohistochemistry, Hohenstein *et al.* (28) claimed an increase of VEGF activity in glomeruli from patients with type 2 diabetes. Bortoloso *et al.* (27), however, found an inverse correlation of glomerular VEGF-A mRNA to albumin excretion rate. Consistent with this finding was the array study from biopsies of patients with DN from Baelde *et al.* (26) showing a decrease in VEGF-A at mRNA and protein levels in glomeruli. Apart from these reports on glomerular VEGF, several groups have shown that glomerular, as well as peritubular capillary rarefaction, represents an important feature of disease progression in DN (28,29) and that the tubulointerstitial changes provide the best prognostic index for progressive DN (3). Thus, both glomerular and tubular VEGF-A may contribute to the maintenance of glomerular or peritubular capillaries and hence tissue survival.

Epidermal growth factor (EGF) is constitutively expressed in the kidney (30). EGF is important for renal tubular cell growth and survival, repair after injury, and regulation of cellular metabolism (30). Several studies demonstrated a progressive decline in EGF urinary excretion in patients with diabetes as well as a reduced EGF expression in experimental DN (31–33). This has been related to the damage and reduction of tubular cells in DN. No data are available for the renal expression of EGF in human DN.

We analyzed molecular markers of potential pathogenic pathways in the interstitium of renal biopsies from patients with DN with emphasis on the growth factors VEGF-A and EGF, as well as matrix components. Our reasons were the discrepant reports on VEGF in DN and that the functional deterioration of DN correlates best with the degree of interstitial disease (34,35). Contrary to rodent models of DN, our study in established human DN revealed a significant decrease of intrarenal VEGF-A expression on mRNA and protein levels, which was associated with a reduction of peritubular capillaries. Thus, in human DN, a lack of VEGF-A, rather than an excess of VEGF-A, may contribute to the progressive disease, raising a note of caution about the proposal to inhibit VEGF-A to prevent progression of DN.

Materials and Methods

Renal Biopsies for mRNA Analysis

Human renal biopsy specimens were procured in an international multicenter study, the European Renal cDNA Bank-Kroener-Fresenius biopsy bank (see the Acknowledgments for participating centers). Biopsies were obtained from patients after informed consent and with approval of the local ethics committees. The characteristics of all patients are shown in Table 1 (36).

For validation of the microarray data, real-time reverse transcriptase–PCR (RT-PCR) analysis of biopsies from patients with progressive DN ($n = 22$) and control subjects (living donor [LD] $n = 9$; deceased donor $n = 1$) were used (Table 1). Additional disease cohorts were 15 patients with membranous glomerulopathy (seven female/eight male; age 63.1 ± 5.0 yr; GFR 68 ± 35 ml/min [range 15 to 115 ml/min]; proteinuria 5.2 ± 2.9 g/d) and seven patients with minimal-change disease (MCD; two female/five male; age 31.7 ± 5.9; GFR 107.8 ± 13.9 [range 84 to 120 ml/min]; proteinuria 6.3 ± 5.6 g/d).

Microdissection and RNA Isolation

After renal biopsy, the tissue was transferred to RNase inhibitor and microdissected into glomerular and tubular fragments. Total RNA was isolated from microdissected tubulointerstitial tissue (for details, see Cohen *et al.* [37]).

Target Preparation

A total of 300 to 800 ng of total RNA was reverse-transcribed and linearly amplified according to a protocol previously reported (36). The fragmentation, hybridization, staining, and imaging were performed according the Affymetrix Expression Analysis Technical Manual.

For microarray analysis, robust multichip analysis was performed. Subsequently, we analyzed the expression arrays with significance analysis of microarrays (38). For more details and for gene expression data of respective probe sets, see http://diabetes.diabetesjournals.org/cgi/content/full/55/11/2993.

Quantitative Real-Time PCR

Reverse transcription and real-time RT-PCR were performed as reported previously (37). Predeveloped TaqMan reagents were used for human EGF (NM_001963), fibronectin 1 (NM_002026), hypoxia-inducible factor 1α (HIF-1α; NM_181054), and the housekeeper genes (Applied Biosystems, Foster City, CA). For VEGF-A, the following oligonucleotide primers (300 nmol/L) and probe (100 nmol/L) were used: Human VEGF-A (NM_003376), sense primer 5′-GCCTTGCTGCTCTACCTCCAC-3′, antisense primer 5′-ATGATTCTGCCCTCCTCCTTCT-3′, and fluorescence labeled probe (FAM) 5′-AAGTGGTCCCAGGCTGCACCCAT-3′. The expression of candidate genes was normalized by two reference genes, 18S rRNA and cyclophilin, giving comparable results. The mRNA expression was analyzed by standard curve quantification.

Pathway Identification

The Ingenuity Pathway Analysis program uses a knowledge base derived from the literature to relate gene products with each other, on the basis of their interaction and function. The differentially expressed probe sets were uploaded to the Ingenuity Pathway Analysis suite to identify networks of genes that are altered by DN.

Computational Promoter Analysis

Promoter regions for VEGF-A and EGF were identified and analyzed using the software tools ElDorado, FrameWorker, and ModelInspector (Genomatix, Munich, Germany; www.genomatix.de). The analyses were performed as described previously by Cohen *et al.* (39).

Immunohistochemistry and Immunofluorescence

For immunohistochemistry, tissue samples were fixed in 4% buffered paraformaldehyde and embedded in paraffin. For clinical data, see Table 2. Target expression was studied by mAb (mouse anti-VEGF, clone VG1; mouse anti-EGF, clone EGF-10; both from Abcam, Cambridge, UK). An avidin-biotin technique was used as reported previously (40).

Table 1. Clinical and histologic characteristics of biopsies from patients with early and established DN, LD, and patients with MCD analyzed by oligonucleotide array-based gene expression profiling and real-time RT-PCR[a]

Biopsy Group	Gender	Age (yr)	Histology Major Diagnosis	Creatinine (mg/dl)	Proteinuria (g/d)	GFR (ml/min)	Hypertension	Diabetes Type	HbA_{1c} (%)	Retinopathy
Established DN										
DN3 (A + P)	M	57	DN	1.6	9.7	48	Yes	1	8.7	Yes
DN5 (A + P)	F	46	DN	2.4	0.4	23	Yes	2	7.5	Yes
DN6 (A + P)	F	67	DN	4.8	2.4	10	Yes	2	NA	Yes
DN9 (A)	M	67	DN	4.0	3.0	16	Yes	2	NA	NA
DN10 (A + P)	M	62	DN	2.3	5.0	39	Yes	2	7.8	No
DN11 (A + P)	M	68	DN	3.5	NA	34	Yes	2	7.2	No
DN16 (P)	M	58	DN	1.7	0.7	44	Yes	2	7.4	No
DN17 (P)	M	77	DN	2.9	3.1	26	Yes	2	NA	No
DN18 (P)	F	59	DN	3.2	21.4	34	Yes	2	NA	NA
DN19 (P)	M	58	DN	2.4	14.2	48	Yes	2	9.8	Yes
DN20 (P)	F	63	DN	3.3	7.0	31	Yes	2	7.9	No
DN21 (P)	M	63	DN	2.1	8.6	51	Yes	2	7.1	No
DN22 (P)	F	47	DN	7.0	NA	9	Yes	2	13.8	Yes
DN23 (P)	M	74	DN	2.5	0.3	39	Yes	2	4.6	No
DN24 (P)	F	63	DN	2.9	6.5	36	Yes	2	6.4	No
DN25 (P)	M	55	DN	2.8	7.5	34	Yes	2	7.8	Yes
DN26 (P)	M	57	DN	1.6	1.4	68	Yes	2	8.8	NA
DN27 (P)	M	63	DN	3.0	0.6	25	Yes	2	6.2	NA
DN28 (P)	M	66	DN	9.8	1.7	6	Yes	2	6.0	No
DN29 (P)	M	60	DN	2.2	1.0	41	Yes	2	6.9	No
DN30 (P)	M	75	DN	1.7	6.2	40	Yes	2	7.3	No
DN31 (P)	F	NA	DN	1.2	10	60	Yes	2	NA	NA
DN32 (P)	F	63	DN	2.5	5.5	40	Yes	2	6.4	NA
mean ± SEM	15/8	62.2 ± 1.7		3.1 ± 0.4	5.5 ± 1.2	34.9 ± 3.3	23/23		7.6 ± 0.5	
Early DN										
DN1 (A)	M	45	DN	0.3	0.7	97	No	2	8.7	Yes
DN2 (A)	F	34	DN	1.4	0.9	51	Yes	1	7.5	Yes
DN4 (A)	M	61	DN	1.1	3.7	85	Yes	2	NA	Yes
DN7 (A)	M	73	DN	1.1	2.4	70	Yes	2	NA	NA
DN8 (A)	M	62	DN	1.2	2.5	54	Yes	2	7.8	No
mean ± SEM	4/1	55 ± 6.9		1.1 ± 0.1	2.0 ± 0.6	71.4 ± 8.8	4/5		8.0 ± 0.4	
LD										
LD1 (A)	F	66	Normal tissue	<1.1	<0.2	>100	No	No	NA	No
LD2 (A)	M	26	Normal tissue	<1.1	<0.2	>100	No	No	NA	No
LD3 (A)	M	49	Normal tissue	<1.1	<0.2	>100	No	No	NA	No
LD4 (P)	F	35	Normal tissue	<1.1	<0.2	>100	No	No	NA	No
LD5 (P)	M	39	Normal tissue	<1.1	<0.2	>100	No	No	NA	No
LD6 (P)	F	55	Normal tissue	<1.1	<0.2	>100	No	No	NA	No
LD7 (P)	M	41	Normal tissue	<1.1	<0.2	>100	No	No	NA	No
LD8 (P)	M	61	Normal tissue	<1.1	<0.2	>100	No	No	NA	No
LD9 (P)	F	58	Normal tissue	<1.1	<0.2	>100	No	No	NA	No
LD10 (P)	M	27	Normal tissue	<1.1	<0.2	>100	No	No	NA	No
LD11 (P)	F	54	Normal tissue	<1.1	<0.2	>100	No	No	NA	No
LD12 (P)	F	61	Normal tissue	<1.1	<0.2	>100	No	No	NA	No
CD5 (P)	NA	NA	Normal tissue	<1.1	<0.2	>100	No	No	NA	No
mean ± SEM	6/6	47.7 ± 4.0		<1.1	<0.2	>100	0/13			
MCD										
MCD1 (A + P)	M	32	MCD	1.3	11.0	104	Yes	No	NA	NA
MCD2 (A + P)	F	32	MCD	0.7	3.0	121	No	No	NA	NA
MCD3 (A + P)	M	16	MCD	1.2	5.4	102	Yes	No	NA	NA
MCD4 (A + P)	M	20	MCD (remission)	0.9	0.2	122	Yes	No	NA	NA
MCD5 (P)	F	57	MCD	1.1	15.0	102	No	No	NA	NA
MCD6 (P)	M	34	MCD	1.4	9.1	84	No	No	NA	NA
MCD7 (P)	M	NA	MCD	0.6	0.6		NA	No	NA	NA
mean ± SEM	5/2	25 ± 4.1		1.0 ± 0.1	4.9 ± 2.3	116.0 ± 5.8	3/7			

[a]A, patients who were analyzed by oligonucleotide array-based gene expression profiling; DN, diabetic nephropathy; HbA_{1c}, glycosylated hemoglobin; MCD, minimal-change disease; P, patients who were analyzed by real-time reverse transcriptase–PCR (RT-PCR).

Table 2. Clinical and histologic characteristics of biopsies from patients with early and established DN and control kidneys analyzed by immunohistologic staining for VEGF, EGF, and PECAM-1/CD31[a]

Biopsy Group	Gender	Age (yr)	Histology Major Diagnosis	Creatinine (mg/dl)	Proteinuria (g/d)	GFR (ml/min)	Interstitial Fibrosis (%)	Interstitial VEGF (%)	Interstitial EGF (%)	Interstitial CD31 (%)	HbA_{1c} (%)	Hypertension	Retinopathy
Early DN													
DN01	M	44	DN	0.9	0.7	150	0	15	10	6	7.1	Yes	No
DN02	M	64	DN	1	0.6	108	0	10	7	12	8	Yes	Yes
DN03	M	48	DN	1	1.5	105	0	8	10	0	7.8	Yes	No
DN04	M	50	DN	1.1	2	80	20	16	12	0	8	Yes	No
mean ± SEM	4/4	51.5 ± 4.3		1.0 ± 0.04	1.2 ± 0.3	110.8 ± 11.0	5.0 ± 5.0	12.0 ± 1.9	10.0 ± 1.0	5.0 ± 2.9	7.7 ± 0.2	4/4	1/3
Established DN													
DN05	M	56	DN	1.2	1.2	64	32	14	10	10	7.4	Yes	Yes
DN06	M	61	DN	1.6	2.1	58	35	12	9	0	7.6	Yes	No
DN07	M	63	DN	0.9	1.7	57	20	10	10	11	5.5	No	No
DN08	M	66	DN	0.8	1	55	28	7	5	4	7.3	Yes	No
DN09	M	59	DN	1.3	1	40	46	4	0	0	5.4	Yes	Yes
DN10	M	79	DN	2.4	1.6	40	22	10	5	6	5.9	No	No
mean ± SEM	6/6	64.0 ± 3.3		1.4 ± 0.2	1.4 ± 0.2	52.3 ± 4.0	31.0 ± 3.9	10.0 ± 1.5	7.0 ± 1.6	5.0 ± 1.9	6.5 ± 0.4	4/6	2/4
Control kidneys													
NK01	M	57	Normal tissue	0.9	0	100	0	84	73	30	NA	Yes	No
NK02	M	60	Normal tissue	0.8	0	98	15	75	67	28	NA	No	No
NK03	M	62	Normal tissue	1	0	96	0	86	78	32	NA	No	No
mean ± SEM	3/3	60.0 ± 1.5		0.9 ± 0.1		98.0 ± 1.2	5.0 ± 5.0	82.0 ± 3.4	73.0 ± 3.2	30.0 ± 5.0		1/3	0/3

[a]PECAM-1, platelet-endothelial cell adhesion molecule-1; VEGF, vascular endothelial growth factor.

For immunofluorescence, frozen, acetone-fixed kidney sections were sequentially hydrated and incubated with the primary monoclonal anti-VEGF or anti-CD31 (clone JC/70A; Abcam), then with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Invitrogen, Milan, Italy). Specificity of antibody labeling was demonstrated by lack of staining after substituting PBS and proper control immunoglobulins (Zymed, Invitrogen, Milan, Italy).

Images were recorded and analyzed using AxioVision software 4.3 and analysis module (Carl Zeiss SpA, Arese, Milan, Italy). Fibrosis and immunoperoxidase staining were evaluated on all acquired images by application of a threshold procedure to highlight selectively the positive areas. The software automatically calculated the percentage of the stained area.

Statistical Analyses

Experimental data are given as means ± SD. Statistical analysis was performed using Kruskall-Wallis, Mann-Whitney *U* tests, *T* test, and Pearson correlation (SPSS 14.0; SPSS, Chicago, IL). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

VEGF-A and EGF Are Repressed in DN

Gene expression was analyzed in the tubulointerstitial compartment from renal biopsies of patients with diabetes and with serum creatinine concentration >1.4 mg/dl (124 μmol/L; $n = 6$) and from patients with early DN (*i.e.*, serum creatinine ≤1.4 mg/dl and only mild histologic alterations of the interstitium; $n = 5$; Table 1). Pretransplantation biopsies from LD ($n = 3$) served as controls with normal renal function. Renal interstitial tissue from patients with MCD ($n = 4$) and normal GFR and interstitial histology but albuminuria was used as a nonprogressive but proteinuric control. The clinical characteristics of the patients are shown in Table 1. Starting from a list of 202 genes, whose gene products might be involved in DN according to common hypotheses (17,41–43) (Supplementary Table S1), we identified 49 genes with significantly altered expression levels in established DN in comparison with controls ($P < 0.05$; Table 3).

Among these genes, the growth factors VEGF (VEGF-A and VEGF-B; VEGF-C was too low expressed to be quantified) and EGF showed a decrease in mRNA expression in DN. For matrix components such as collagens I and IV, fibronectin 1, and vimentin as well as matrix-modulating enzymes (matrix metalloproteinase-2, -7 and -14 and tissue inhibitor of metalloproteinase 1 and 3), an increase of mRNA levels was observed. In biopsies with incipient DN, only four genes, namely E47-like factor 1, matrix metalloproteinase-7, platelet-endothelial cell adhesion molecule (PECAM-1)/CD31, and von Willebrand factor were significantly regulated. Importantly, VEGF-A and EGF showed already at this stage of DN a tendency to decreased mRNA expression, albeit not statistically significant.

Pathway Analysis Demonstrated Central Role for Both Growth Factors

The Ingenuity Pathway Analysis suite identifies dynamically generated biologic networks on the basis of current literature knowledge. We used this bioinformatic tool to generate functional networks of the genes that are differentially regulated in DN. The respective network is given in Figure 1. It contains 35 genes (18 genes from the regulated gene list). VEGF-A and EGF showed, in contrast to most other genes, a decrease in expression in DN. Both have central positions in the generated network, underlining the important role of these factors. The striking finding of reduced expression of VEGF-A and EGF and their central physiologic role prompted us to study further their expression in DN.

Validation of Microarray Results with Quantitative RT-PCR

Quantitative RT-PCR was used to validate the Affymetrix results in an independent cohort of patients with DN. Real-time

Table 3. Microarray gene expression data of the 49 regulated genes[a]

Gene Symbol	Probe Set ID	Fold Change	Q Value (%)	Gene Title
Upregulated genes				
ADAM10	214895_s_at	2.9121	0.1055	ADAM metallopeptidase domain 10
ADAM10	202603_at	1.6379	1.2069	ADAM metallopeptidase domain 10
ADAM17	213532_at	1.4811	0.9700	ADAM metallopeptidase domain 17 (tumor necrosis factor, α, converting enzyme)
ADAM9	202381_at	1.8185	0.0755	ADAM metallopeptidase domain 9 (meltrin γ)
AKR1B1	201272_at	1.9109	0.4272	Aldo-keto reductase family 1, member B1 (aldose reductase)
BMP1	202701_at	1.2063	0.5045	Bone morphogenetic protein 1
BMP6	206176_at	1.4837	3.8646	Bone morphogenetic protein 6
CASP1	211366_x_at	2.2426	0.0440	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, β, convertase)
CASP1	209970_x_at	2.3725	0.0440	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, β, convertase)
CASP1	211368_s_at	2.6279	0.0440	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, β, convertase)
CCL21	204606_at	1.5204	1.9243	Chemokine (C-C motif) ligand 21
COL1A2	202404_s_at	2.7623	0.2614	Collagen, type I, α2
COL4A1	211980_at	3.0281	0.0440	Collagen, type IV, α1
COL4A1	211981_at	2.8670	0.0440	Collagen, type IV, α1
COL4A2	211966_at	2.1982	0.0440	Collagen, type IV, α2
COL4A2	211964_at	2.0089	0.1055	Collagen, type IV, α2
ELF1	212418_at	1.61281	0.0755	E74-like factor 1 (ets domain transcription factor)
ELF2	203822_s_at	1.2898	1.2069	E74-like factor 2 (ets domain transcription factor)
ELF3	210827_s_at	1.7153	0.5974	E74-like factor 3 (ets domain transcription factor, epithelial-specific)
ELK3	221773_at	3.2305	0.0440	ELK3, ETS-domain protein (SRF accessory protein 2)
ERG	213541_s_at	1.2747	3.8646	v-ets erythroblastosis virus E26 oncogene like (avian)
ETS2	201328_at	1.7125	0.3487	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)
FLII	222065_s_at	1.2336	0.5974	Flightless I homolog (*Drosophila*)
FN1	211719_x_at	4.0309	0.0440	Fibronectin 1
FN1	216442_x_at	3.1158	0.0440	Fibronectin 1
FN1	212464_s_at	3.4687	0.0440	Fibronectin 1
FN1	210495_x_at	3.2295	0.0440	Fibronectin 1
ICAM-1	202637_s_at	1.4090	0.1055	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor
ICAM-1	202638_s_at	1.5242	0.1709	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor
ICAM-2	213620_s_at	1.7471	1.9243	Intercellular adhesion molecule 2
MET	203510_at	1.8486	0.0440	Met proto-oncogene (hepatocyte growth factor receptor)
MET	211599_x_at	1.4021	0.5045	Met proto-oncogene (hepatocyte growth factor receptor)
MGC5618	221477_s_at	2.2802	0.1709	Hypothetical protein MGC5618
MMP-14	160020_at	1.2018	0.7642	Matrix metallopeptidase 14 (membrane-inserted)
MMP-14	202827_s_at	1.2150	1.9243	Matrix metallopeptidase 14 (membrane-inserted)
MMP-2	201069_at	1.2837	1.9243	Matrix metallopeptidase 2 (gelatinase A, 72-kD gelatinase, 72-kD type IV collagenase)
MMP-7	204259_at	14.2773	0.0440	Matrix metallopeptidase 7 (matrilysin, uterine)
NFKB-1	209239_at	1.2086	0.2131	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)
PECAM-1	208982_at	2.7041	0.0440	Platelet/endothelial cell adhesion molecule (CD31 antigen)
PECAM-1	208981_at	1.8652	0.0440	Platelet/endothelial cell adhesion molecule (CD31 antigen)
PECAM-1	208983_s_at	2.2719	0.0440	Platelet/endothelial cell adhesion molecule (CD31 antigen)
PLAT	201860_s_at	1.5406	3.8646	Plasminogen activator, tissue
SOD2	215223_s_at	2.3740	1.9243	Superoxide dismutase 2, mitochondrial
SPP1	209875_s_at	1.5568	0.7642	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)
TGFBI	201506_at	2.9495	0.0440	Transforming growth factor, β -induced, 68 kD
TGFBR2	208944_at	2.4363	0.0440	Transforming growth factor, β receptor II (70/80 kD)
TIMP1	201666_at	4.6579	0.0440	TIMP metallopeptidase inhibitor 1
TIMP3	201149_s_at	1.4667	0.2796	TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)
TIMP3	201147_s_at	1.2611	1.2069	TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)
VCAM-1	203868_s_at	2.1310	0.5045	Vascular cell adhesion molecule 1
VIM	201426_s_at	3.1098	0.0440	Vimentin
VWF	202112_at	3.4252	0.0440	Von Willebrand factor
Downregulated genes				
ADRA2B	208544_at	0.7954	1.2069	Adrenergic, α-2B-, receptor
AGT	202834_at	0.4087	0.3487	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
BMP1	206725_x_at	0.8025	1.9243	Bone morphogenetic protein 1
EGF	206254_at	0.3369	0.0440	Epidermal growth factor (β-urogastrone)
ERF	203643_at	0.6636	0.0440	Ets2 repressor factor
KNG1	206054_at	0.5691	0.5974	Kininogen 1
MMP17	206234_s_at	0.8136	1.5560	Matrix metallopeptidase 17 (membrane-inserted)
SPDEF	213442_x_at	0.8680	0.9700	SAM pointed domain containing ets transcription factor
TGFBR3	204731_at	0.4899	0.1308	Transforming growth factor, β receptor III (betaglycan, 300 kD)
VEGF	212171_x_at	0.6642	0.0440	Vascular endothelial growth factor
VEGF	210512_s_at	0.4452	0.0440	Vascular endothelial growth factor
VEGF	210513_s_at	0.7016	0.1308	Vascular endothelial growth factor
VEGFB	203683_s_at	0.7699	1.5560	Vascular endothelial growth factor B

[a]Comparison of a list of 202 genes, which might be involved in DN, revealed 49 genes with significantly altered expression levels in DN compared with controls ($P < 0.05$). Thirty-eight genes were upregulated, and 11 were downregulated.

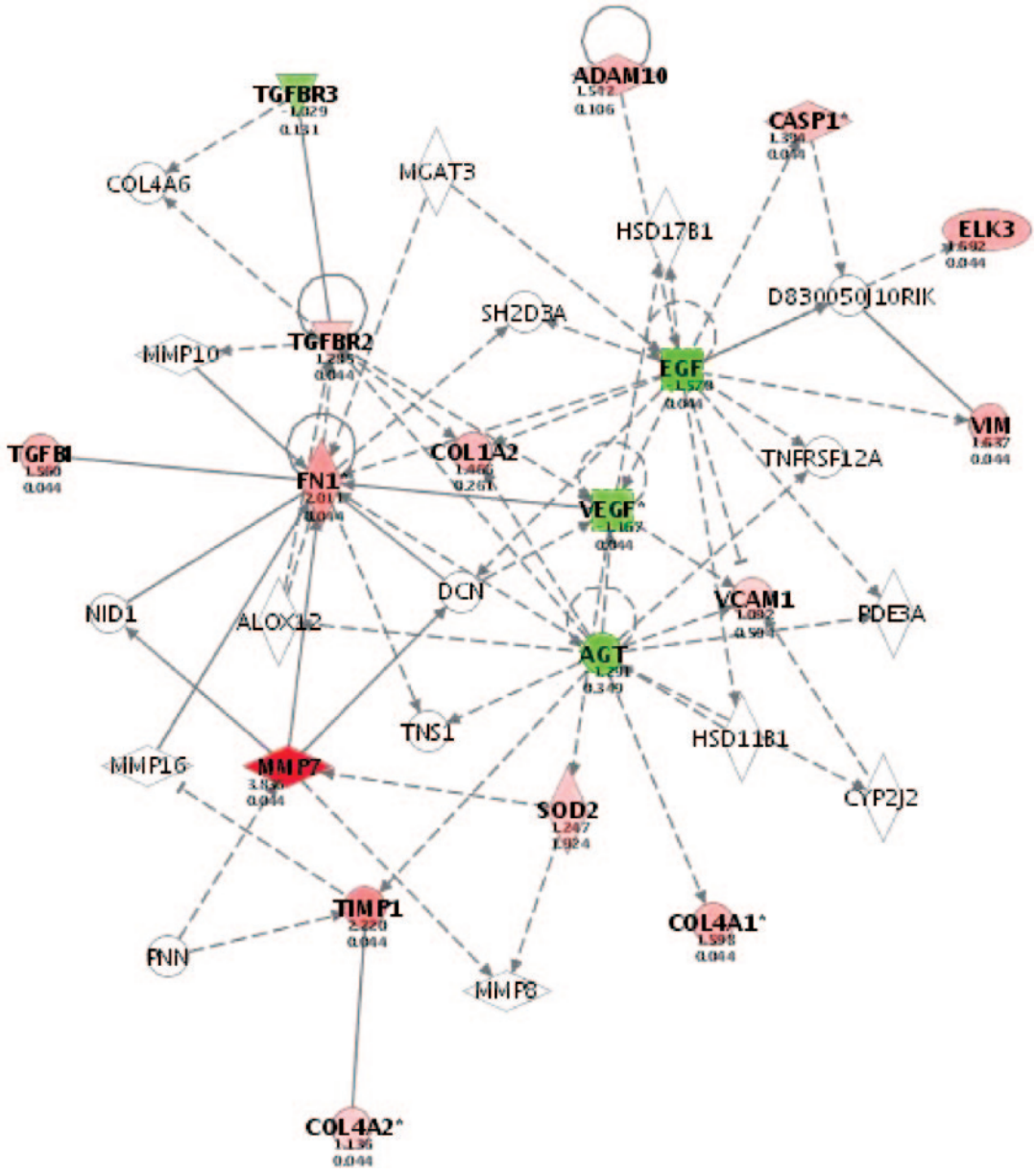

Figure 1. Connectivity map of the responses by Ingenuity Pathway analysis. The colored symbols mark the input genes, where red indicates an upregulation and green a downregulation of the specific gene.

RT-PCR was performed on microdissected tubulointerstitium of 22 DN biopsy samples and on 10 donor kidney samples that served as control group (Table 1). In Figure 2, the results for VEGF-A, EGF, and fibronectin mRNA expression are shown. VEGF-A and EGF were significantly downregulated in the tubulointerstitium of biopsies with DN compared with the control group (Figure 2, A and B), whereas fibronectin showed an increase in mRNA production (Figure 2C). Thus, the RT-PCR analyses confirmed the results of the microarray analysis.

To investigate whether other renal diseases also may lead to reduced VEGF-A mRNA, we analyzed its expression in a cohort of 15 patients with membranous glomerulonephropathy (MGN). Like DN, MGN is a common progressive and proteinuric renal disease that presents with glomerular changes that lead to subsequent interstitial lesions. In addition, patients with MCD as a proteinuric disease without significant tubulointerstitial disease were studied ($n = 7$). Neither MGN nor MCD showed the reduction of tubulointerstitial VEGF-A expression that was seen in DN (mean ± SD: LD 1 ± 0.22, DN 0.30 ± 0.21 [$P < 0.001$], MGN 0.58 ± 0.60 [NS], MCD 0.62 ± 0.39 [NS]; range of VEGF-A expression: LD 0.75 to 1.33, DN 0.02 to 0.83, MGN 0.30 to 2.70, MCD 0.13 to 1.26).

To exclude the possibility that age of the patient has an effect on VEGF-A expression in the tubulointerstitium, we performed correlation analysis with age. In 10 kidney donors (range of age 27 to 61 yr), no correlation of age and mRNA expression for VEGF-A could be seen. Furthermore, the entire cohort showed no association of age with VEGF-A ex-

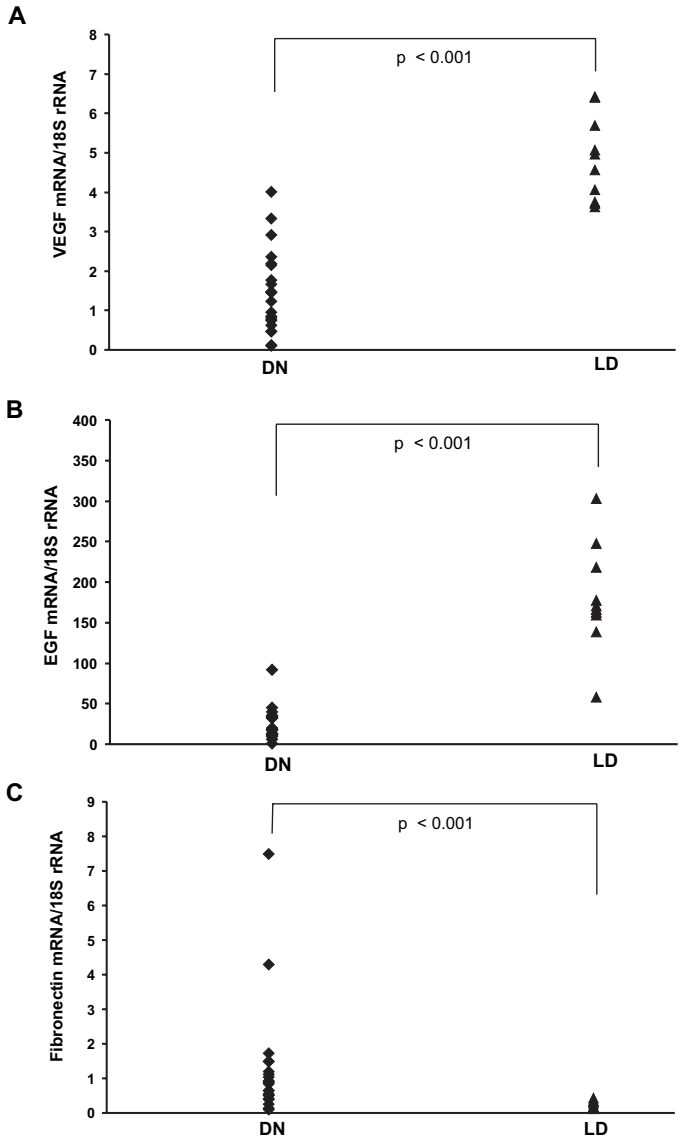

Figure 2. Confirmation of the array expression pattern of three genes by real-time reverse transcriptase–PCR normalized to 18S rRNA. mRNA expression of vascular endothelial growth factor A (VEGF-A; A), EGF (B), and fibronectin (C) was quantified in microdissected renal biopsies from control tissues (living donor [LD], $n = 9$; deceased donor, $n = 1$) and biopsies from patients with established diabetic nephropathy (DN; $n = 22$). All three genes were significantly ($P < 0.001$) regulated compared with control samples, independent of the housekeeper gene used for normalization (18S rRNA, cyclophilin). The graphs show expression ratios of each gene to 18S rRNA.

pression (53 biopsies from individuals with DN, MCD, MGN, or LD).

Because the expression of VEGF-A may be differentially regulated in the glomerular and tubular compartment of kidneys with DN, the expression of VEGF-A and EGF was studied by real-time RT-PCR on microdissected glomeruli from biopsies with DN and controls. As was shown in the tubulointerstitium, mRNA levels of VEGF-A and EGF were reduced in glomeruli of DN biopsies compared with LD controls (VEGF-A: LD 1 ± 1.03, DN 0.39 ± 0.22 [$P < 0.05$]; EGF: LD 1 ± 1.73, DN 0.17 ± 0.24 [$P < 0.05$]).

Relationship among VEGF-A and EGF Reduction, Capillary Rarefaction, and Tubular Damage

To evaluate whether the lower mRNA levels correspond to decreased protein levels, we performed immunohistochemical staining for VEGF-A and EGF on fixed renal biopsies (established DN, $n = 6$; early DN, $n = 4$; controls, $n = 3$). In the control group, VEGF-A and EGF were detected in epithelial cells of proximal and distal tubules. In patients with DN, a marked and consistent decrease in VEGF-A (Figure 3A) and EGF (Figure 3B) staining was observed, confirming the mRNA data on the protein level. Concomitantly, epithelial flattening, increased thickness of the tubular basement membrane, and overall interstitial damage were seen. By immunofluorescence on frozen sections (established DN, $n = 6$; early DN, $n = 4$; controls, $n = 3$), the repression of VEGF-A in DN compared with controls could also be confirmed (Figure 4A). The staining for both factors showed a general trend to be negatively correlated with fibrosis as determined by semiquantitative grading ($r = -0.46$ for VEGF-A [NS] and -0.48 for EGF [NS], respectively). The staining for both growth factors showed a positive correlation with each other ($r = 0.99$, $P < 0.001$).

The density of the peritubular and interstitial vessels was evaluated because VEGF-A is an important angiogenic factor. As a marker for endothelial cells, we used PECAM-1/CD31 (44). A clear reduction of PECAM-1/CD31 staining was observed in biopsies with DN compared with controls (Figure 4B). Especially small peritubular vessels were reduced, whereas larger vessels were still positive, consistent with peritubular capillary rarefaction.

To determine whether the decreased VEGF-A and EGF mRNA expression is just a reflection of decreased volume of tubular cells, we explored the expression of a variety of additional tubular gene products as well as other relevant genes, such as EGF and VEGF receptors (see Supplementary Table S2). The well-evaluated majority of these genes showed no reduced expression, indicating that tubular atrophy or rarefaction is an unlikely cause for the clear reduction of VEGF-A and EGF.

Potential Mechanism of mRNA Regulation

Steady-state mRNA levels reflect a balance between transcription and turnover. Comparative promoter analysis was performed for VEGF-A and EGF to identify potential similarities in the proximal promoter of VEGF-A and EGF (39). No similarities that could explain co-regulation could be identified between their promoters.

To investigate further the reduced *in vivo* expression of VEGF-A and EGF in DN, we performed experiments to examine the potential influence of factors that previously were implicated in gene regulation in DN *in vitro* (albumin and glucose [14,45]). Neither albumin nor glucose had a significant effect on VEGF-A expression in human tubular cells (Supplementary Table S3).

Regulation of VEGF-A occurs not only at the transcriptional level but also posttranscriptionally (46). Regulation of VEGF-A includes mRNA stabilization by Hu antigen R (HuR; or ELAV

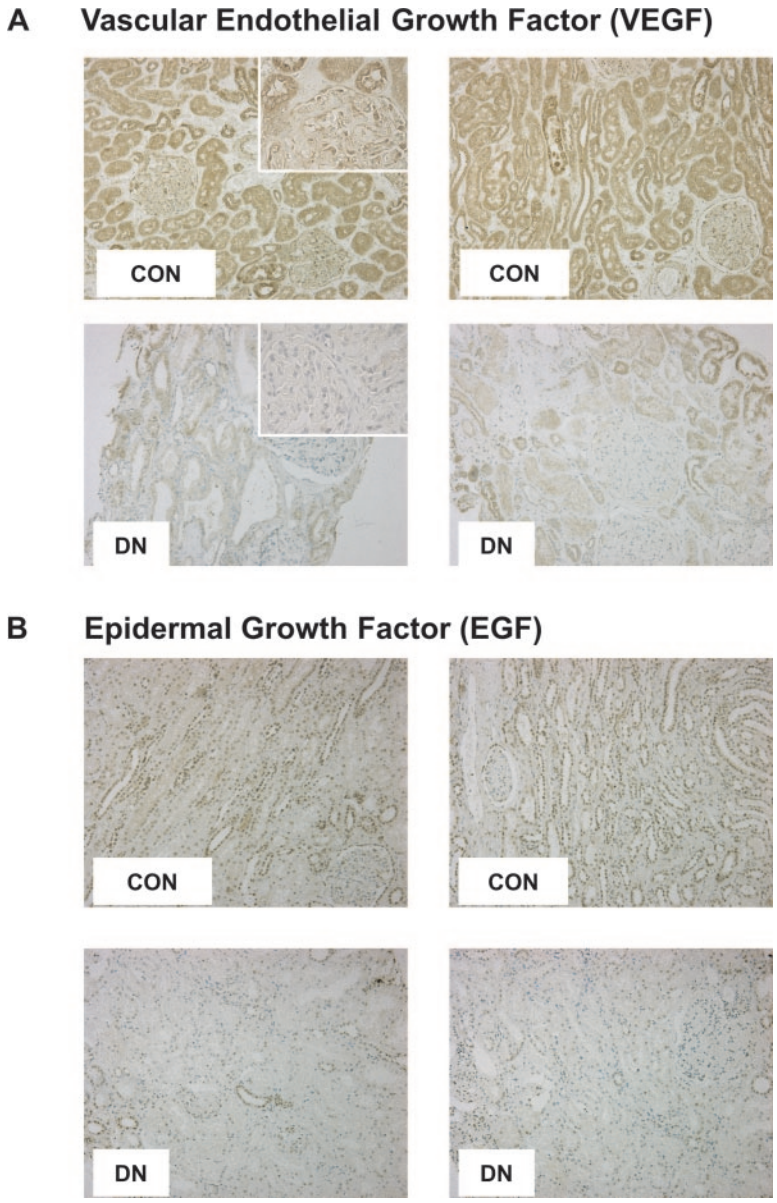

Figure 3. Immunohistochemistry and immunofluorescence for VEGF-A and EGF in tubulointerstitial compartment of human renal biopsies. Immunohistochemical analysis for VEGF (A) and EGF (B) showed that both growth factors were constitutively expressed in the tubulus in control samples. In patients with DN, there was a significant decrease in immunostaining for both proteins (immunoperoxidase). (Insets) Higher magnification show the expression of VEGF by glomerular and tubular epithelial cells. In DN, VEGF-A staining is reduced in epithelial cells of both renal compartments (immunoperoxidase). Magnifications: ×100 in A and B; ×630 in insets.

[embryonic lethal, abnormal vision]), a ubiquitously expressed RNA-binding protein (46). To determine whether HuR mRNA stabilization might be active in DN, we analyzed RNA expression of known HuR targets (47) in the DN microarray expression profiles. Of the HuR targets examined, only VEGF-A mRNA was reduced. This suggests that HuR-dependent RNA degradation is an unlikely mechanism for VEGF reduction.

HIF-1α is the main regulator of VEGF-A expression, mainly by posttranslational regulation *via* proteasomal degradation and stabilization by von Hippel-Lindau factor. To study mRNA expression of HIF-1α, we performed real-time RT-PCR, because the expression was too low to be quantified on the microarray. A significant decrease of HIF-1α mRNA expression (normalized to 18S rRNA; $P < 0.01$) could be detected in patients with DN compared with the control samples. Furthermore, mRNA levels of HIF-1α and VEGF-A showed a positive correlation ($r = 0.47$, $P < 0.01$).

Correlation of mRNA Levels of VEGF-A with Proteinuria

To evaluate potential relationships between clinical and experimental data, we performed correlation analysis. A significant inverse correlation ($r = -0.34$, $P < 0.05$) between VEGF-A

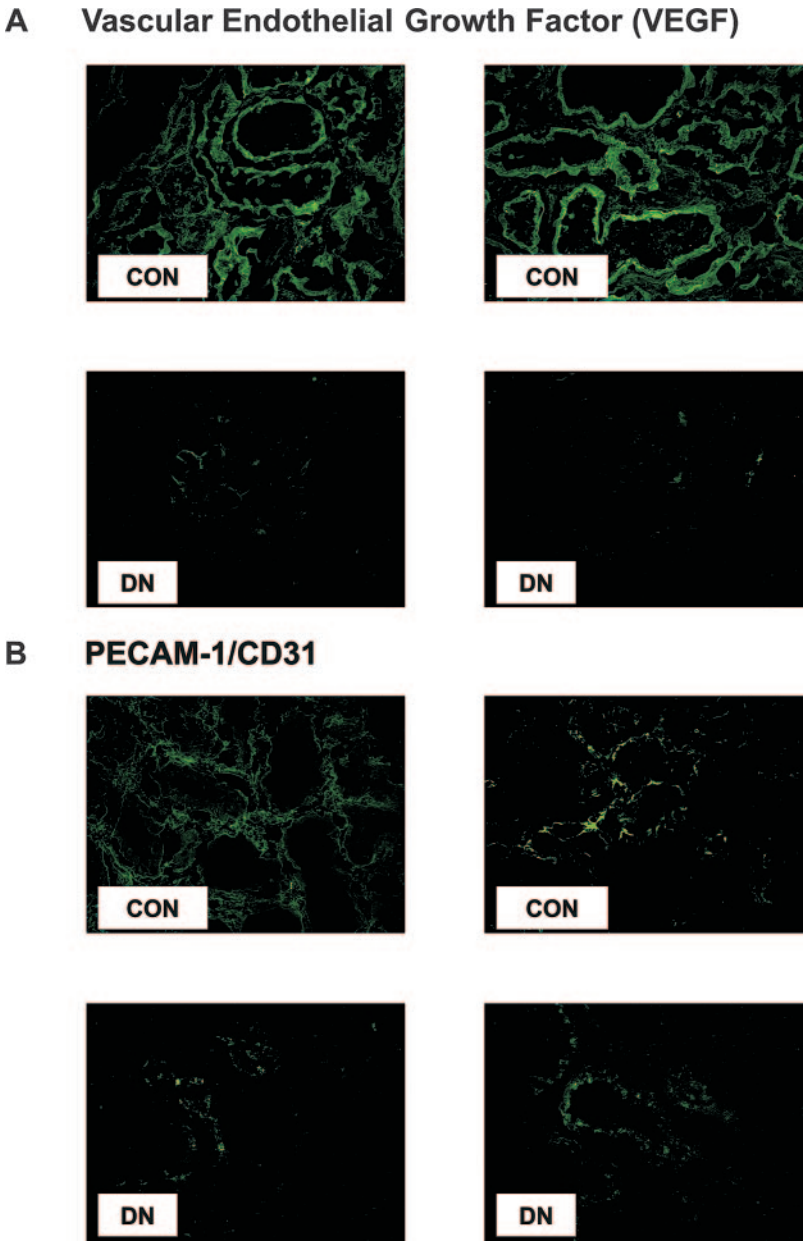

Figure 4. Immunofluorescence for VEGF-A and platelet-endothelial cell adhesion molecule (PECAM-1)/CD31 in biopsies of control subjects and patients with DN. (A) Immunofluorescence for VEGF showed a constitutive expression in the control group, whereas in patients with DN, a significant decrease of staining intensity was observed. (B) Immunostaining for PECAM-1/CD31 in the renal interstitium demonstrated the endothelial marker expressed in small vessels and peritubular capillaries in the control group, whereas for patients with DN, PECAM-1/CD31 expression was clearly reduced (immunofluorescence). Magnifications: ×200 in A; ×250 in B.

mRNA levels and proteinuria was found in DN, suggesting that a decrease in VEGF-A expression may be involved in progressive proteinuria, or *vice versa*. A similar correlation pattern was found for EGF and proteinuria ($r = -0.34$, $P < 0.05$). No such correlation was seen in patients with other diseases (MCD and MGN).

Discussion

We observed a decrease in VEGF-A expression by both mRNA and immunohistologic analysis in the tubulointerstitial compartment of biopsies from patients with DN. In addition, we found reduced expression of glomerular VEGF-A mRNA in renal biopsies of DN. These results are in contrast to the increased renal VEGF-A expression described in some rodent models of DN (9,12–14). In human diabetic retinopathy, VEGF-A is increased (16,17), suggesting that diabetic retinopathy and DN may have a different pathophysiology. This is supported by differences in microvascular changes: Retinopathy is characterized by neoangiogenesis (16); DN shows microvascular rarefaction (28,29). The increased VEGF-A that is seen in some animal models of experimental DN has led to the hypothesis that human DN may be due to overexpression of

VEGF-A. Our findings of reduced renal tubulointerstitial VEGF-A expression together with a rarefaction of peritubular capillaries suggest a different conclusion.

The apparent contradictory results between rodent and human could be because rodents demonstrate only very early signs of DN but do not develop progressive DN (25). The initial biopsies that were examined in this study were derived from patients with established DN, so elevated VEGF-A expression early in human DN could have been missed. We therefore examined renal biopsies from patients with early DN before the development of renal failure. However, even in these biopsies, a tendency for reduced VEGF-A expression was also observed. Therefore, differences in the stages of DN may not be a sufficient explanation for the differences in VEGF-A expression between human and rodent DN.

The decreased VEGF-A levels that were observed correlate with a reduction in peritubular microvasculature. A reduced microvasculature in kidney of DN is also supported by earlier studies (29). Thus, reduced VEGF-A may contribute to decreased endothelial survival and angiogenesis, a speculation that rests primarily on the correlative data and the biology of VEGF-A in angiogenesis. In this context, it is of interest that Chung *et al.* (48) reported reduced VEGF-A and microvasculature in arterial tissue from patients with diabetes. This occurred together with upregulation of angiostatin production. It was proposed that angiostatin synthesis reduced VEGF-A formation, causing a reduction in capillary density in diabetes. Taken together, this would result in the microvascular disease of diabetes. Such a scenario could also apply to DN and would be consistent with our results. However, no evidence for an induction of angiostatin expression could be found in the DN samples studied.

To examine potential factors that contribute to reduced VEGF-A levels, we examined proteinuria, because it inversely correlated with VEGF-A expression in the patients with DN. It has been proposed that proteinuria may contribute to tubulointerstitial injury and progression of renal injury (49). We therefore examined VEGF-A expression in biopsies from patients with MGN and MCD. VEGF-A mRNA expression was reduced in some of these patients, but the overall VEGF-A expression was not significantly lower than that seen in control subjects, suggesting that proteinuria cannot explain the reduced VEGF-A expression.

VEGF-A mRNA stabilization is mediated in part by the mRNA-binding protein HuR (46,47). Our results did not show co-regulation of HuR-dependent genes with VEGF-A.

A correlation between HIF-1 and VEGF-A mRNA expression was observed in this study. HIF-1α is expressed in glomerular podocytes and tubular epithelial cells and is a major regulator of VEGF-A (50). Because HIF-1α mRNA-levels were decreased, we analyzed the mRNA of a subgroup of HIF-regulated genes to evaluate the transcriptional activity of HIF. No reduction of mRNA expression was observed for any of these genes, with exception of VEGF-A (data not shown). A generalized reduction of HIF-1α transcriptional activity therefore does not seem to be a cause for the VEGF-A reduction in DN.

The mammalian kidney, especially tubular cells, is a major site of constitutive EGF synthesis (30). Previous studies in patients with various degrees of DN reported a decline in urinary EGF excretion as DN progressed (31,33). In this study, we observed a decrease in tubulointerstitial EGF mRNA expression in kidney biopsies of patients with DN. Tubular epithelial cells show varying degrees of cellular alterations and flattening in DN. EGF both is produced by and acts as a growth factor on these epithelial cells. Therefore, a primary lack of EGF production might contribute to their pathology in DN, or the damage of tubular cells during diabetes might lead to reduced EGF synthesis, causing a vicious cycle.

Pathway analysis software was used to generate a functional network of genes that link VEGF-A and EGF. The results also suggest a central role for these factors in DN. Because EGF is a tubular survival hormone and VEGF-A is a capillary survival factor, a decrease in EGF expression could lead to a reduction of tubular cells, as shown by Kelly *et al.* (32), which in turn could cause a reduction of VEGF-A expression. Furthermore, reduced VEGF-A expression may lead to tubular damage by ischemia and so to a decrease of EGF. Unfortunately, our results cannot distinguish which might be the initial factor.

Conclusion

Human DN is associated with a reduced renal expression of VEGF-A and EGF, a decrease in renal microvascular density, and tubular epithelial atrophy. These findings point toward a lack of VEGF-A and EGF as potential factors that contribute to DN by microvascular rarefaction and tubular atrophy. This may result in hypoxia-ischemia factors constituting progression factors for DN. Therefore, enhancement of VEGF generation (*e.g.*, by HIF stabilization) and preservation of the renal microvasculature would be therapeutic goals. This is in contrast to the suggested therapeutic inhibition of VEGF on the basis of results of some DN rodent models. Angiotensin-converting enzyme inhibitors or AT-1 antagonists, current nephroprotective therapies in DN, may already involve protection of the peritubular microvessels. In the context of DN, our data indicate that multiple factors that are implicated in the progression of DN may in fact be interrelated (*e.g.*, the renin-angiotensin system, hypoxia, ischemia, HIF, VEGF). The molecular mechanisms of this interplay have yet to be elucidated. Concluding from our results, potentially inhibiting the renin-angiotensin system in combination with enhancing VEGF expression could represent a future therapeutic avenue for protection from DN.

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opsy bank at the time of the study: C.D. Cohen, M. Fischereder, H. Schmid, P.J. Nelson, M. Kretzler, D. Schlöndorff, Munich; J.D. Sraer, P. Ronco, Paris; M.P. Rastaldi, G. D'Amico, Milano; F. Mampaso, Madrid; P. Doran, H.R. Brady, Dublin; D. Mönks, C. Wanner, Würzburg; A.J. Rees, P. Brown, Aberdeen; F. Strutz, G. Müller, Göttingen; P. Mertens, J. Floege, Aachen; N. Braun, T. Risler, Tübingen; L. Gesualdo, F.P. Schena, Bari; J. Gerth, G. Wolf, Jena; R. Oberbauer, D. Kerjaschki, Vienna; B. Banas, B.K. Krämer, Regensburg; W. Samtleben, Munich; H. Peters, H.H. Neumayer, Berlin; K Ivens, B. Grabensee, Düsseldorf; R.P. Wüthrich, Zürich; V. Tesar, Prague.

Disclosures

None.

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