Fibrogenesis in progressive renal disease

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Chapter 7

Renal cortical and glomerular message for angiogenic factors is decreased in human diabetic nephropathy

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Abstract

Vascular complications in diabetic patients are being increasingly appreciated as a significant clinical challenge. Experiments in animal models indicate that perturbed angiogenesis and corresponding alterations of expression of angiogenic growth factors play a role in the development of diabetic nephropathy (DN). We investigated expression levels of angiogenic factors in human DN in relation to fibrosis and the number of endothelial cells.

Cortical and glomerular mRNA levels for vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF), transforming growth factor-beta (TGF-β), fibroblast growth factor 2 (FGF2) and syndecan were measured using real-time PCR in biopsy material from 29 patients with DN and in 24 control samples. Laser capture microdissection was applied to obtain glomerular RNA. Interstitial fibrosis was quantified with Sirius Red staining and the number of endothelial cells by CD31 staining.

Both cortical and glomerular VEGF and CTGF mRNA levels were decreased in DN compared to control samples ($P<0.01$). Expression of TGF-β, FGF2 and syndecan was not significantly different between diabetic patients and controls. Glomerular mRNA levels for VEGF showed an inverse correlation with the extent of interstitial fibrosis and a positive association with CTGF expression and the number of endothelial cells.

The results are suggestive for a disturbed angiogenesis in patients with DN. We hypothesize that decreased secretion of VEGF and CTGF due to damage of glomerular epithelial cells in DN leads to a disturbed maintenance of glomerular endothelial cells and a loss of glomerular and interstitial capillaries.

Introduction

Diabetic nephropathy (DN) is a major complication of both type 1 and type 2 diabetes. It is the most common single cause of end-stage renal disease (1,2). One of the earliest clinical signs of diabetic nephropathy is microalbuminuria, which may progress to proteinuria (3) and hyperfiltration followed by a decrease in the glomerular filtration rate (GFR). Morphologically, DN is characterized by glomerular hypertrophy, progressive expansion of the mesangial matrix, and thickening of the glomerular and tubular basement membranes (4,5). In later stages of the disease, glomerulosclerosis and interstitial fibrosis, resulting from an imbalance between synthesis and degradation of extracellular matrix (ECM) components (6), are prominent.

Although many factors including high glucose, insulin, advanced glycation end products (AGE) and high blood pressure have been found to play a role in the initiation and progression of DN, the precise molecular mechanism is still unclear. Several cytokines and growth factors have been proposed to mediate the development of DN. Transforming growth factor-beta (TGF-β) is the most widely investigated molecule in relation to diabetic glomerulosclerosis and interstitial fibrosis, most notably in animal models (7). Vascular endothelial cell dysfunction is a common finding in patients with type 2 diabetes mellitus. Endothelial cell loss may be involved in the development of renal disease and the progression to sclerosis. In diabetic retinopathy, neovascularization can result in loss of vision. Increased VEGF protein has been demonstrated in nonvascular cells in the retina of patients with diabetes even in the absence of retinopathy (8). A role for VEGF in DN has also been described. In STZ-induced diabetic rats, it has been demonstrated that treatment with monoclonal anti-VEGF antibodies decreased hyperfiltration, albuminuria, and glomerular hypertrophy (9). Others studies have reported that VEGF is reduced in rat glomeruli one week after diabetes induction with streptozotocin (10). In human DN it has been suggested that VEGF is important in maintaining glomerular endothelial cell function, and that a decrease in local VEGF levels accounts for abnormal remodelling of the glomerular capillaries (11,12). Despite recent progress in elucidating the role of perturbed vascular biology in progressive renal disease, the role of VEGF in the pathogenesis of DN is still unclear (13).

Several other growth factors have been identified, which play a role in angiogenesis. For example, CTGF has been shown to play an important role in invasive new vessel formation, in addition to its profibrotic activity downstream of TGF-β. A similar involvement has been described for FGF2 (14). Syndecan can amplify the availability of FGF2 via specific binding to FGF2 (15).

In this study we investigated the gene expression levels of different angiogenic factors in renal biopsies from patients with DN. These factors have been identified in a previous study of gene expression profiling of human kidneys with DN (11). Correlations of expression levels

with clinical parameters and morphological changes were tested to obtain further insights in the role of these mediators in the development of DN.

Materials and methods

Array studies on diabetic kidneys

Hybridizations were performed on the Human Genome U95Av2 Array (Affymetrix® Santa Clara, CA, USA). This array contains ~12,000 sequences characterized previously in terms of function or disease association. Ten µg of total RNA from three control kidneys and three kidneys from patients with diabetic nephropathy (11) was converted to complementary (c)DNA, double stranded (ds)DNA, and transcribed *in vitro* according to the instructions of the manufacturer. After hybridization, the microchips were scanned and analyzed with Affymetrix® Microarray Suite 5.0 software. To normalize the data from different microarray experiments, the expression levels of all genes on the chip were scaled to a standard value and the mean of the scaling factors was calculated. This value served as the normalization factor for all genes represented on the different microarray chips. To obtain normalized expression values, the expression level for each gene was multiplied by the normalization factor. Statistics of this method can be found in the Microarray Suite User's Guide, Version 5.0, which is available at http://www.affymetrix.com/support/technical/manuals.affx.

To identify genes of which expression was altered consistently in the disease samples, we used either those genes which were present on all six chips, or those which were present in all three control samples and absent in all three diabetic samples, or vice versa. We employed multiple pair-wise comparisons between control and disease groups using the OpenStat statistics package (students t-test, $p<0.01$).

Renal biopsies

Frozen biopsies from patients with DN (n=29) were selected from the pathology archive of the Leiden University Medical Center and the Institute for Clinical Pathology, Heidelberg. All patients were diagnosed with type 2 diabetes. Diabetic nephropathy was histologically confirmed by Periodic acid-Schiff (PAS) stained paraffin sections. Criteria for diabetic nephropathy included glomerular hypertrophy, diffuse mesangial and focal nodular glomerulosclerosis, arteriolar hyalinosis, focal and segmental glomerulosclerosis, the presence of hyaline drops between Bowman's capsule and epithelial cells, and interstitial fibrosis. Serum creatinine levels and albuminuria of the patients are summarized in Figure 1. As a control group (n=22), native kidneys with normal function and histology were obtained at autopsy (n=3), from cadaver donor kidneys unsuitable for transplantation for technical reasons (n = 7), and

from the non-affected part of tumour nephrectomy samples (n = 12). There were no significant differences within the different control groups between all parameters measured. Patient studies were approved by the institutional ethical committee.

Microdissection and RNA isolation from glomeruli

The PALM Laser-MicroBeam System (P.A.L.M., Wolfratshausen, Germany) was used for microdissection of glomeruli from renal biopsies according to the method of Specht et al (16). In brief, three frozen sections of 4 µm were put on a polyethylene foil coated slide. After selecting the glomeruli (20 ± 5 glomeruli from each biopsy), they were dissected with a laser beam and catapulted into a reaction tube containing RLT buffer (RNeasy kit, QIAGEN GmbH, Germany). RNA was isolated from these glomeruli by adding of 200µl Trizol®. After adding 40 µl chloroform and mixing for 1 min, the suspension was centrifuged at 15,000g for 10 min. RNA was precipitated with isopropanol in the presence of 5 µg glycogen.

RNA isolation from renal cortex

Cryostat sections were cut from each biopsy. The presence of cortex was determined on the basis of light-microscopic localization of glomeruli in the sections. Ten slides of 10 µm thickness were cut and collected in a reaction tube. RNA was isolated using RNeasy Mini columns (QIAGEN GmbH, Germany), according to the instructions of the manufacturer. After elution, the RNA was precipitated with 3 M NaAc and ethanol in the presence of 5 µg glycogen.

cDNA synthesis

RNA from glomerular and whole kidney was converted into cDNA using avian myeloblastosis virus reverse transcriptase (Roche Applied Science) in combination with oligo-dT and random hexamer (0.5 µg/reaction) priming. RNA without

Table 1 . Primer and Taqman® probes sequences.

Gene	Forward primer	Reverse primer	Taqman® probe
VEGF	AAACCCTGAGGGAGGCTCC	TACTTGCAGATGTGACAAGCCG	CCTCCTGCCCGGCTCACCGC
CTGF	GGAAGAGAACATTAAGAAGGGCAA	CTCGGTATGTCTTCATGCTGGTG	CGTACTCCCAAAATCTCCAAGCCTATCA
TGF-β1	CCCAGCATCTGCAAAGCTC	GTCAATGTACAGCTGCCGCA	ACACCAACTATTGCTTCAGCTCCACGGA
Fibronectin	GGAGAATTCAAGTGTGACCCTCA	TGCCACTGTTCTCCTACGTGG	AGGCAACGTGTTACGATGATGGAAGACAT
FGF-2	CGACGGCCGAGTTGACGG	CAGGTAACGGTTAGCACACACTCC	AAGAGCGACCCTCACATCAAGCTACAACTTCA
Syndecan	GAGCTGCGTGTCCTTCCAAG	CCCAGAGACCAACGTTCAAGC	TGCCCCCTGAAGATCAAGATGGCTC
GAPDH	TTCCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG	CCCAGCCTTCTCCATGGTGGTGAA
HPRT	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT	CTTGACCATCTTTGGATTATACTGCCTGACCA
TBP	CACGAACCACGGCACTGATT	TTTTCTTGCTGCCAGTCTGGAC	TGTGCACAGGAGCCAAGAGTGAAGA

reverse transcriptase was used as a negative control for the PCR.

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (Q-PCR) (17) was used in combination with Taqman® probes to quantify the gene transcription levels for VEGF, TGF-β, CTGF, FGF2, and syndecan. Primer and probe sequences can be found in Table 1. In brief, the cDNA samples were diluted 25 times. Five µl was used for quantitation of each transcript. Each PCR consisted of the following components: 300 pmol primers, 100 pmol probe 0.25 mM dNTPs, 6 mM $MgCl_2$, 0.5 U of Hot Gold Star polymerase, 1 x real-time PCR buffer (Eurogentec). Further details concerning the real-time PCR protocol have been described elsewhere (18). The transcription levels of the genes of interest were determined and corrected to a panel of three housekeeping genes, i.e., glyceraldehyde-phosphate-dehydrogenase (GAPDH), hypoxanthine phosphoribosyl transferase (HPRT), and TATA box binding protein (TBP), as described by Vandesomple et al (19). These housekeeping genes were selected from a panel of 5 different housekeeping genes (20) on basis of the highest correlations between each other ($r \geq 0.85$). To calculate the relative mRNA levels we measured the threshold cycle (Ct) values of a standard curve with a known amount of total RNA. For each housekeeping gene the relative amount of the samples were calculated by linear regression analysis from their standard curve. The relative values of each of the 3 different housekeeping genes of the controls were adjusted to one by dividing the samples by the mean of all samples. After this correction the mean of the 3 different housekeeping genes was calculated. The relative expression levels of the genes of interest

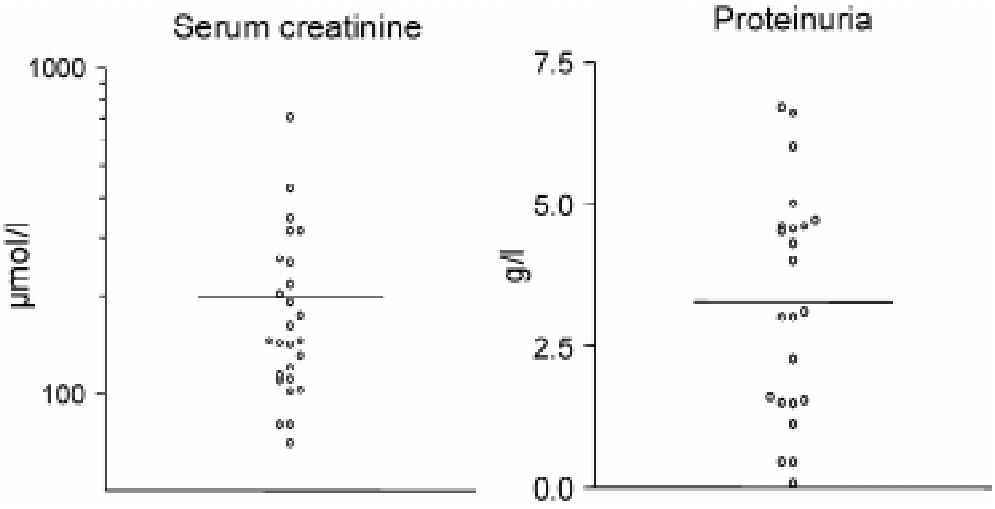

Figure 1. Clinical characteristics of the diabetic patients. Serum creatinine levels (µmol/l) (A) and urine protein excretion (g/l) (B).

were calculated by dividing the value of the gene by the mean of the different housekeeping genes. The relative values were set to one for the controls.

(Immuno)histochemistry

To quantify the number of endothelial cells, frozen section were stained for CD31. In brief, the slides were washed in PBS and incubated for 2 h at room temperature with the primary antibody (Rabbit anti CD31, DAKO, Glostrup, Denmark), diluted in 1% bovine serum albumin in PBS. After washing with PBS, the slides were incubated for 30 min with horseradish peroxidase–conjugated anti-rabbit Envision (DAKO, Glostrup, Denmark). The slides were again washed with PBS, and the staining was developed with diaminobenzidine. The color was enhanced by rinsing the slides in 0.5% $CuSO_4$ solution for 5 min. After counterstaining with haematoxylin, the slides were dehydrated and mounted. Sirius Red staining was used to quantify the amount of interstitial fibrosis. All sections were stained in one session simultaneously.

Digital image analysis

Digital image analysis was performed using a Zeiss microscope equipped with a full-color 3CCD camera (Sony DXC 950p) and KS-400 image analysis software version 3.0 (Zeiss-Kontron, Eching, Germany). The percentage of the area stained for CD31 was calculated and used as a measure for the number of endothelial cells. To count the number of glomerular endothelial cells, images of all of the glomeruli (3-15) present in the slides were taken. For the interstitial endothelial cells 10 random images were taken. To quantify the amount of interstitial fibrosis the percentage of the area in the sections stained by Sirius Red was measured in 10 adjacent microscopic fields of the renal cortex.

Statistics

Statistical analysis for comparison of mRNA expression, Sirius Red stained area and CD31 stained area, between groups was performed using independent Student's T-test. Pearson correlations were calculated using the SPSS version 10 software. Values of $p<0.05$ were considered to be significant.

Results

Microarray experiments

In earlier gene expression profiling experiments on isolated glomeruli from patients with DN, a significant decrease of mRNA levels for VEGF (19.5 times) and CTGF (6.5 times) was found (11). To further expand the scope of these investigations, we have performed oligonucleotide

microarray profiling of RNA isolated from the total cortical part of kidneys from the same patients as those described in the previous study. A total of 6 chips were hybridized with RNA from 3 controls and 3 patients with diabetic nephropathy. Results of these experiments can be found in detail at : http://www-onderzoek.lumc.nl/pathology/kidney/diabeticnephropathy/ and http://www.ncbi.nlm.nih.gov/geo/gds/gds browse.cgi?gds=961. Whole cortex mRNA levels of VEGF (2.2 times), CTGF (1.7 times), and syndecan (2.2 times) were decreased, while expression of fibronectin (FN) (1.7 times) was increased in patients with DN compared to controls. There were no significant differences between DN and control tissue with respect to TGF-β mRNA levels.

Clinical parameters

Protein excretion and serum creatinine levels at the time of biopsy for the 29 patients with DN are summarized in Figure 1. Serum creatinine levels ranged from 70 to 700 µmol/l (Fig 1A) and protein excretion ranged from 0.061 to 6.7 gram/l (Fig 1B). There was no difference in age (60.6 ± 10.9 years versus 59.9 ± 10.0 years) and gender between control and diabetic patients.

Glomerular and whole cortical mRNA levels.

Having identified transcripts as being differentially regulated in DN using oligonucleotide microarrays, we validated their mRNA levels in a large, independent group of 29 patients and 22 controls. VEGF mRNA levels in microdissected glomeruli as measured by Q-PCR were 2.6 times decreased ($P<0.01$) in patients with DN compared to control kidneys (Fig 2A). In whole cortex RNA, VEGF mRNA levels in patients with DN were decreased 2.5 fold compared to controls ($P<0.01$) (Fig 3A). A similar pattern of downregulation was observed for CTGF. Glomerular and whole cortex mRNA levels for CTGF were 1.6 times decreased ($P<0.05$) in patients with DN (Fig 2B) (Fig 3B). There were no significant differences in TGF-β mRNA levels between controls and patients with DN in both glomerular and whole kidney RNA (Fig 2C and 3C). There was a slight increase in TGF-β mRNA levels (1.3 fold) in whole kidney RNA from patients with DN compared to controls. This difference was not significant ($P= 0.103$).The levels for FN mRNA in patients with DN were 2.8 times increased in glomerular samples ($P<0.05$) (Fig 2D) and 1.9 times increased in whole cortex ($P<0.05$) (Fig 3D), compared to controls. FGF2 and syndecan whole cortex mRNA levels were not significantly different between diabetic patients and controls (Fig 3E and 3F).

(Immuno)histochemistry

Interstitial fibrosis was quantified by morphometric analysis on frozen sections stained with Sirius Red. There was a significant increase in the extent of Sirius Red staining in patients with

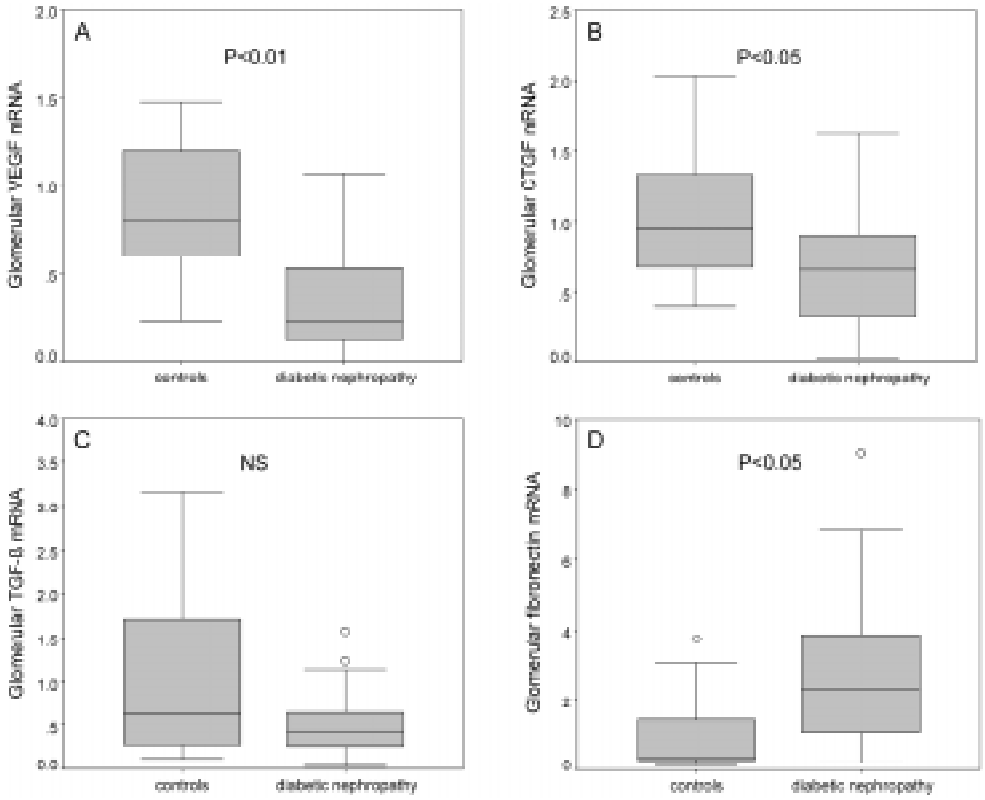

Figure 2. Box and whisker plots of glomerular mRNA levels. The boxes contain 50% of the values. The upper and lower border indicate the 25th and the 75th percentile. The upper and lower whisker indicate the highest and lowest value. The black line in the box indicates the median and open circles indicate outliers. Relative glomerular mRNA levels for VEGF (P<0.01) (A), CTGF (P<0.05) (B), TGF-β (not significant) (C), and fibronectin (P<0.05) (D).

DN compared to controls (33.3% versus 18.7%) (Fig 5 A). The numbers of CD31 positive cells are quantified by measuring the CD31 positive area. Both in the glomerular (Fig 5B) and tubulo-interstitial (Fig 5C) area of patients with DN there was a significant decrease in the extent of CD31 staining compared to controls (7.0% versus 22.6% for the glomeruli and 5.8% versus 9.9% in the tubulo-interstitial part), suggesting a reduction in the number of capillaries/ endothelial cells in DN. Representative pictures of these stainings can be found in Fig 4.

Correlations

To identify statistically significant relationships between variables, Pearson correlations were calculated between clinical, histological, and mRNA levels. Results of these correlations are summarized in Table 2. There was a positive correlation between glomerular VEGF and CTGF

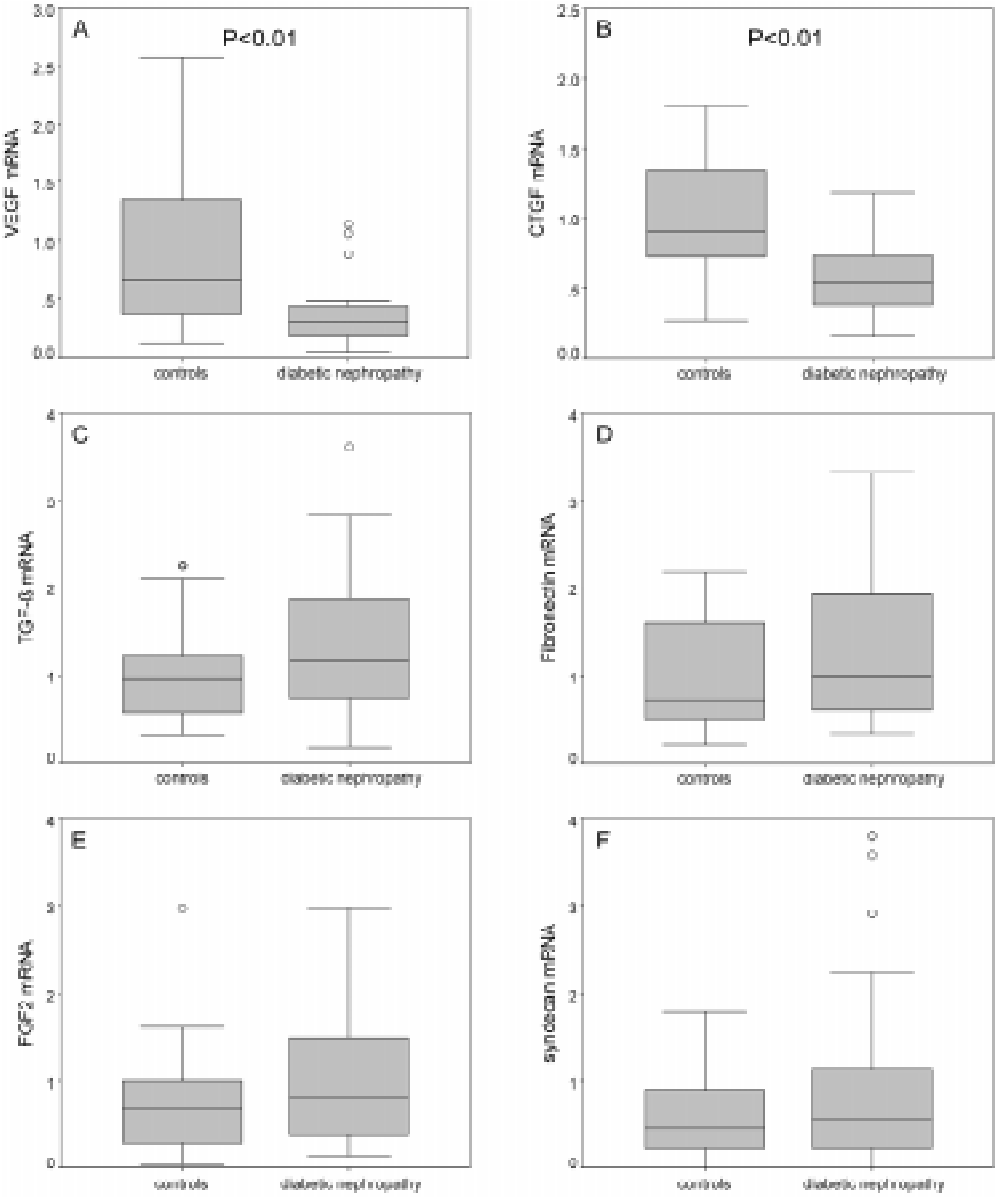

Figure 3. Box and whisker plots of whole cortex mRNA levels. Relative tubulo-interstitial mRNA levels for VEGF (P<0.01) (A), CTGF (P<0.01) (B), TGF-β (not significant) (C), fibronectin (P<0.05)(D), FGF2 (not significant)(E), and syndecan 1 (not significant) (F).

mRNA levels within the diabetic patients (R=0.61, P<0.01). A negative correlation was found for glomerular VEGF mRNA levels and Sirius Red positive area (R=-0.62, P<0.01). In other words,

Table 2. Pearson correlations.

					Interstitial mRNA level						Interstitial		Glomerular	Glomerular mRNA level			
			Serum creat.	Prot.	FGF2	VEGF	TGF-β	CTGF	FN	Syndecan	Sirius Red	CD31	CD31	VEGF	TGF-β	CTGF	FN
	Serum creat.	correlation (R)	1.000														
		P-value	.														
	Prot.	correlation (R)	0.462	1.000													
		P-value	0.030	.													
Interstitial	FGF2	correlation (R)	0.001	0.133	1.000												
mRNA		P-value	0.997	0.566	.												
level	VEGF	correlation (R)	-0.223	-0.259	0.017	1.000											
		P-value	0.274	0.245	0.926	.											
	TGF-β	correlation (R)	-0.062	0.313	0.568	-0.022	1.000										
		P-value	0.763	0.157	0.001*	0.904	.										
	CTGF	correlation (R)	0.007	0.443	0.400	0.095	0.518	1.000									
		P-value	0.974	0.044	0.020	0.598	0.002*	.									
	FN	correlation (R)	0.016	0.483	0.302	-0.033	0.447	0.342	1.000								
		P-value	0.936	0.023	0.099	0.857	0.010*	0.060	.								
	syndecan	correlation (R)	-0.137	-0.428	-0.221	0.089	-0.206	-0.127	-0.365	1.000							
		P-value	0.503	0.047	0.216	0.617	0.249	0.481	0.040	.							
Interstitial	Sirius Red	correlation (R)	0.191	0.287	0.208	-0.233	-0.001	0.022	-0.138	-0.381	1.000						
		P-value	0.383	0.234	0.319	0.251	0.995	0.916	0.510	0.055	.						
	CD31	correlation (R)	0.472	0.334	-0.334	0.201	-0.278	-0.223	0.247	0.172	-0.123	1.000					
		P-value	0.019	0.150	0.089	0.304	0.160	0.263	0.215	0.382	0.548	.					
Glomerular	CD31	correlation (R)	0.265	0.149	-0.309	0.531	-0.233	-0.044	0.149	0.296	-0.455	0.675	1.000				
		P-value	0.222	0.541	0.125	0.004*	0.252	0.831	0.467	0.133	0.019	0.000*	.				
Glomerular	VEGF	correlation (R)	-0.066	0.011	-0.462	0.328	-0.261	-0.135	-0.132	0.020	-0.618	-0.021	0.327	1.000			
mRNA		P-value	0.765	0.963	0.020	0.102	0.208	0.521	0.530	0.924	0.001*	0.923	0.119	.			
level	TGF-β	correlation (R)	-0.137	-0.173	0.084	-0.121	0.382	0.264	0.025	0.513	-0.105	-0.261	-0.291	-0.204	1.000		
		P-value	0.525	0.466	0.683	0.548	0.054	0.193	0.902	0.006*	0.616	0.207	0.158	0.317	.		
	CTGF	correlation (R)	-0.220	0.089	-0.147	0.196	0.045	0.263	-0.108	0.035	-0.255	-0.256	-0.134	0.611	0.158	1.000	
		P-value	0.301	0.709	0.474	0.328	0.829	0.194	0.599	0.864	0.219	0.217	0.522	0.001*	0.432	.	
	FN	correlation (R)	-0.229	0.189	0.046	-0.105	0.394	0.320	0.308	-0.072	-0.143	-0.128	-0.171	-0.037	0.385	0.291	1.000
		P-value	0.293	0.439	0.826	0.610	0.052	0.119	0.134	0.726	0.506	0.550	0.424	0.862	0.052	0.149	.

* P< 0.01

there was a relationship between the amount of interstitial fibrosis and the reduction of glomerular VEGF mRNA. Glomerular mRNA levels for VEGF also correlated with glomerular nephrin mRNA levels ($R=0.57$, $P<0.01$, data not shown). Whole cortex mRNA measurements showed correlations between TGF-β and CTGF ($R=0.52$, $P<0.01$), between TGF-β and FN ($R=0.45$, $P=0.01$), between TGF-β and FGF-2 ($R=0.57$, $P<0.01$), and between VEGF and glomerular CD31. When evaluating all samples together (controls and DN), a significant correlation was found between VEGF and the CD31 positive area both for the glomerular ($R=0.50$, $P<0.01$) and the tubulo-interstitial compartments ($R=0.42$, $P<0.01$) (data not shown).

Discussion

In the present study mRNA levels of genes involved in angiogenesis were studied in the renal cortex and in microdissected glomeruli in renal biopsies from patients with DN and healthy controls. We found downregulation of VEGF and CTGF. These levels were related to serum levels of creatinine, the degree of albuminuria, the degree of interstitial fibrosis, and the number of endothelial cells.

The genes measured in this study were identified using microarray analysis on glomerular and tubulo-interstitial mRNA from diabetes patients with DN and healthy controls. Many genes were differentially expressed (see: http://www-onderzoek.lumc.nl/pathology/kidney/diabeticnephropathy/). These lists of differentially expressed transcripts showed several genes that may play a role in endothelial cell maintenance and angiogenesis. To explore the implications of these gene expression alterations, a number of these genes were further investigated in a larger group of patients with different stages of disease, so that the roles of these growth factors in each phase of the disease could be further investigated.

VEGF is one of the most important angiogenic growth factors regulating vasculogenesis and permeability of endothelial cells. Our results show decreased VEGF mRNA levels in patients with DN. Quantification of VEGF mRNA levels in biopsies from patients with DN was shown for the first time by Bailey et al (21). Using *in situ* hybridization, they found a decrease in VEGF mRNA in glomeruli from patients with DN. These results were subsequently confirmed by others in both the interstitial (22) and the glomerular compartments (12). In addition, a correlation was found between glomerular VEGF levels and proteinuria. Results from different animal studies on the action of VEGF are contradictory. In streptozotocin (STZ)-induced diabetic rats, treatment with anti-VEGF antibodies decreased hyperfiltration, albuminuria, and glomerular hypertrophy (23). Tumstatin peptide, an inhibitor of angiogenesis, suppressed glomerular matrix expansion, the development of albuminuria, and renal mRNA expression of VEGF in STZ-induced diabetic mice (24). Other studies report that glomerular expression of VEGF is

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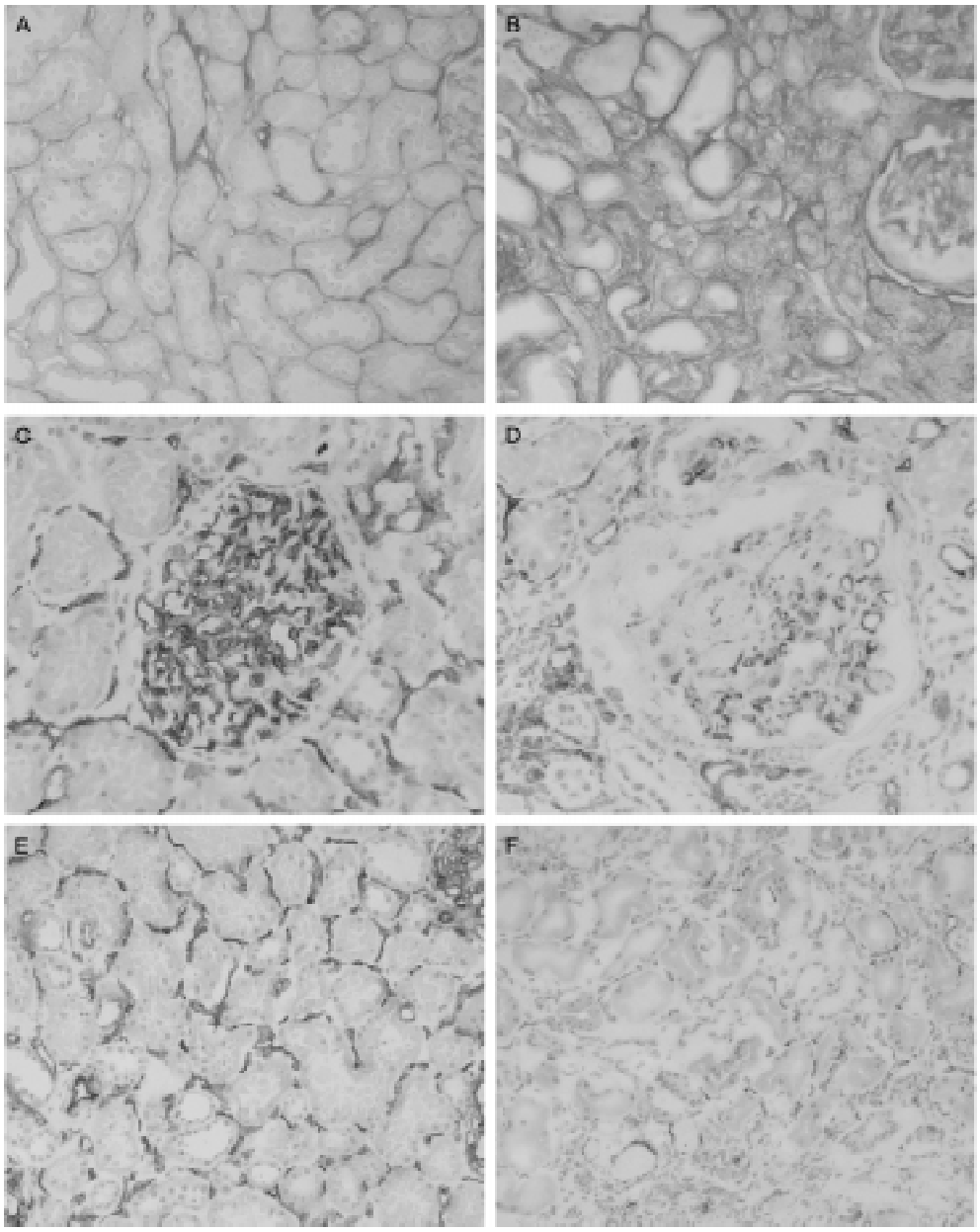

Figure 4. Sirius Red and CD31 staining in the biopsies**.** A and B are representative illustrations of the Sirius Red staining in a control patient and in a patient with DN, respectively. C-F are representative pictures of CD31 staining: glomerulus of a control patient (C), glomerulus of a patient with DN (D), the tubulo-interstitial part of a control patient (E) and the tubulo-interstitial part of a diabetic patient (F).

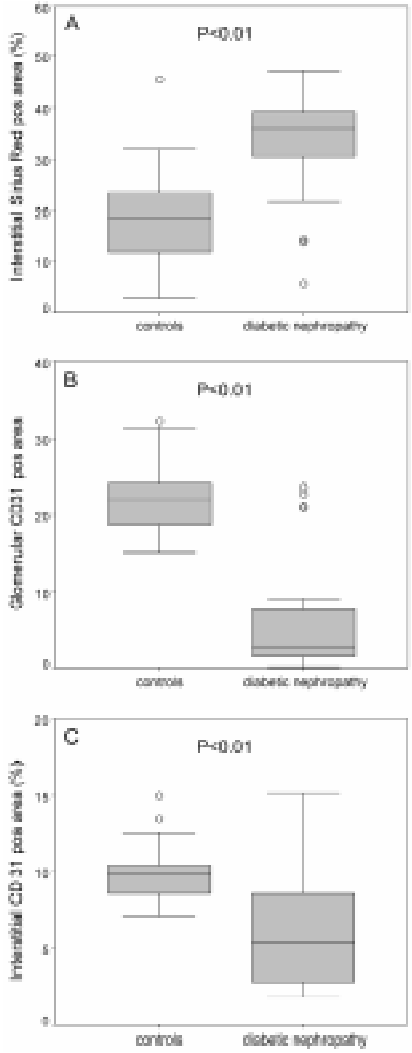

Figure 5. Box and whisker plots of Sirius Red and CD31 staining in the biopsies. (A) The extent of interstitial fibrosis measured as the percentage of the interstitial Sirius Red positive area in the renal cortex. Diabetic patients showed significantly more fibrosis than controls (P<0.01). (B) Percentage of glomerular CD31 staining. (C) Percentage of tubulo-interstitial CD31 staining. The percentage of CD31 positive area in both the glomerular and tubulo-interstitial compartments in patients with DN were significantly decreased compared to controls (P< 0.01).

reduced one week after STZ-induction. This reduction could be restored by treatment of the rats with insulin (10). Decreased VEGF expression was recently documented in the remnant kidney model, and treatment of these animals with VEGF reduces renal fibrosis (25). Studies in mice show that glomerular-selective depletion or overexpression of VEGF-A leads to glomerular abnormalities (26).

In our study CTGF mRNA expression levels were downregulated in both the glomerular and tubulo-interstitial regions. Our findings are in contrast with those from other studies. With suppression subtractive hybridization, the expression of CTGF was found to be increased in mesangial cells under high glucose conditions (27). Ito et al showed an increase in CTGF expression in human biopsies with different progressive renal diseases including DN (28). An increase of CTGF mRNA has also been found in glomeruli from patients with type 1 diabetes (29). However, these data represented the number of mRNA molecules per glomerulus while our data represents relative steady state mRNA levels corrected for three different housekeeping genes. In our studies we did not find increased expression of TGF-β in DN, although in the tubulo-interstitial mRNA samples there was a slight but non-significant increase in TGF-β mRNA levels. This small increase is in line with studies on human glomeruli in which a small increase in TGF-β expression has also been found (30,31), while a key role for TGF-β has been described in animal models of DN (7).

Fibronectin, one of the major extracellular matrix proteins present in glomerulosclerotic and interstitial fibrotic lesions, showed an increase in patients with DN. Although there was no significant increase for TGF-β, a correlation was found between interstitial

TGF-β and FN mRNA within the diabetic patient group. Similarly, interstitial CTGF and TGF-β mRNA showed a significant correlation with each other. This observation corresponds with previous studies that indicate that CTGF might act as a downstream mediator of TGF-β bioactivity (32). The observation that expression of FN is upregulated in DN, while the expression of TGF-β and that of CTGF are not may be explained by studies demonstrating that high glucose can induce FN independent of TGF-β1(33) and CTGF (34).

mRNA levels measured in our study correspond in broad lines with those described in the literature, especially with what has been reported in humans. The discrepancies between the expression levels reported in animal models and those in our study can be explained by several factors. Firstly, it should be noted that animal models do not completely mimic the physiological state of patients with diabetes, and it is likely that mechanisms for initiation and progression of DN in animals differ from those seen in humans (35). Another reason for the differences between our findings and those reported in the literature may be that the stage of the diseases studied is different. Animal models are often studied in an early phase of the disease while most of our patients were already in a moderate to severe stage of the disease. In patient studies, it is often difficult to receive biopsy material at an early stage of disease, as patients do not normally present to a nephrologist until the disease has become manifest.

To obtain more insight into the role of the studied molecules during the progression of DN, we quantified the amount of interstitial fibrosis in the biopsies with Sirius Red. Because we know that histological grading of tubulo-interstitial fibrosis is closely correlated with the loss of renal function (36), quantitative measurements of the Sirius Red staining were used as a morphological marker for renal function. We found a significant increase in the amount of Sirius Red staining in patients with DN compared to controls. The observation that VEGF mRNA levels negatively correlated with the amount of Sirius Red staining support the concept that loss of VEGF is related to progression of the disease. This was already suggested by Bortoloso et al (12) who found a negative correlation between VEGF and urinary protein excretion. There was a positive correlation between glomerular mRNA levels of CTGF and VEGF. This suggests that CTGF, normally present in the podocyte, in combination with other angiogenic factors, may contribute to the normal maintenance of glomerular endothelial cells.

We have also examined whether there is a correlation between the reduced mRNA levels of angiogenic factors and the number of endothelial cells. The extent of CD31 staining, a marker for endothelial cells, was quantified. Both the glomeruli and whole cortex showed a significant decrease in the CD31 positive area in DN indicating a reduction in the number of endothelial cells. This reduction correlated with the reduced VEGF mRNA levels, indicating an association between the number of endothelial cells and the angiogenic growth factor VEGF.

The question remains as to the precise mechanism accounting for the down-regulation of

VEGF and CTGF mRNA in DN. One explanation may be that specific down regulation occurs in a diabetic milieu. This is unlikely since cell culture experiments have shown that glucose, insulin, and advanced glycated end products (AGEs) increase levels of VEGF and CTGF (37-40) . It is more likely that the decrease in expression of angiogenic factors, normally produced by podocytes, results from podocyte loss. The number of podocytes decreases over the course of DN (41,42). Decreased expression in patients with DN of several podocyte specific genes such as nephrin, Wilms tumour 1 (WT1), and GLEPP1, in our glomerular gene profile supports this hypothesis.

From the results of this study we speculate that loss of angiogenic factors contributes to the progression of DN. Podocyte injury in response to diabetes leads to loss of the slit diaphragms and proteinuria. The resulting podocyte loss then leads to a reduction in expression of angiogenic factors, which are necessary for the normal maintenance of the endothelial cells. The reduced endothelial cell maintenance in combination with endothelial cell dysfunction leads to a loss of endothelial cells followed by a loss of glomerular capillaries. This hypothesis is corroborated by the finding that patients with POEMS (Crow-Fukase) syndrome, who have elevated plasma VEGF levels, in combination with Type 2 diabetes do not develop DN (43). The role of VEGF in the maintenance of endothelial cells is also supported by the finding that soluble Flt1 (fms-like tyrosine kinase 1), a splice variant of the major VEGF receptor Flt1, can induce proteinuria in preeclampsia (44). Soluble Flt1 acts as a strong antagonist of VEGF. Increased levels of circulating sFlt1 are associated with decreased circulating levels of VEGF, and administration of soluble Flt1 to pregnant rats results in proteinuria and glomerular endotheliosis (44). Loss of endothelial cells can lead to thrombotic microangiopathy followed by loss of capillaries. Development of the latter has been shown by fragmented red blood cells, and PAI-1, a marker for progression of thrombosis to sclerosis, in glomeruli from diabetic patients with severe nodular lesions (45).

For the observed tubulo-interstitial down regulation of VEGF, we found supporting evidence in the hypothesis of Kang et al (13) that reduction of peritubular capillaries results in impaired delivery of oxygen and nutrients to the tubules and interstitial cells leading to ischemia, cytokine-induced proliferation of fibroblasts, and ECM synthesis. Loss of peritubular capillaries in interstitial fibrosis has been observed in human renal diseases (46) and in several models of interstitial fibrosis.

In conclusion, this study demonstrates a reduction in mRNA levels of angiogenic factors in glomerular and whole kidney mRNA samples from renal biopsies of patients suffering from type 2 diabetes induced DN. Reduction of these factors correlated with the extent of interstitial fibrosis. We also found a reduction of glomerular and interstitial endothelial cells measured with CD31 staining in DN. These results may suggest an important role for angiogenic factors

in the maintenance of endothelial cells. Reduced expression of angiogenic factors may be involved in the progression of DN.

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