Fibrogenesis in progressive renal disease

Baelde, J.J.

Chapter 6

Gene expression profiling in glomeruli from human kidneys with diabetic nephropathy

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Abstract

Diabetic nephropathy is a frequent complication in patients with diabetes mellitus. To find improved intervention strategies in this disease, it is necessary to investigate the molecular mechanisms that are involved. To obtain more insight in the processes that lead to diabetic nephropathy, mRNA expression profiles of diabetic glomeruli and glomeruli from healthy individuals were compared.

Two morphologically normal kidneys and two kidneys from patients with diabetic nephropathy were used for the study. Glomerular RNA was hybridized in duplicate on Human Genome U95Av2 Arrays (Affymetrix®). Several transcripts were further tested in independent patient groups and at the protein level by immunohistochemistry.

Ninety-six genes were upregulated in the diabetic glomeruli, whereas 519 genes were downregulated. The list of over expressed genes in diabetic nephropathy includes aquaporin 1, calpain 3, hyaluronoglucosidase, and platelet/endothelial cell adhesion molecule (PECAM-1). The list of downregulated genes includes bone morphogenetic protein 2 (BMP-2), vascular endothelial growth factor (VEGF), fibroblast growth factor 1 (FGF-1), insulin-like growth factor binding protein (IGFPB-2), and nephrin. A decrease in VEGF and nephrin could be validated at the protein level and also at the mRNA level in renal biopsies of 5 additional diabetes patients.

In conclusion, the results of oligonucleotide microarray analyses on control and diabetic glomeruli are presented and discussed in their relation to vascular damage, mesangial matrix expansion, proliferation, and proteinuria. Our findings suggest that progression of diabetic nephropathy might result from a diminished tissue repair capability.

Introduction

Diabetic nephropathy (DN) is a major cause of morbidity in patients with type II diabetes (1). One of the earliest clinical signs of diabetic nephropathy is microalbuminuria, which often progresses towards proteinuria (2). Characteristic features associated with diabetic nephropathy include hyperfiltration, followed by a decrease in the glomerular filtration rate (GFR), glomerular hypertrophy, progressive expansion of the mesangial matrix, and thickening of the glomerular and tubular basement membranes (3,4). These features may precede the development of glomerulosclerosis and interstitial fibrosis, and eventually the onset of end-stage renal disease.

Little is known about the molecular mechanisms leading to end-stage renal disease in diabetic nephropathy. While the role of many genes in progressive renal diseases has been described (5,6), their interrelationship remains largely unclear. With the completion of the human genome project and the development of microarray technology it is now possible to simultaneously screen the RNA expression of thousands of genes in healthy and diseased organs, or in parts of them. Although gene profiling studies have been described recently in animal models for diabetic nephropathy (7), microarray studies on isolated glomeruli from human diabetic kidneys have not yet been reported.

In this study, we investigated the gene expression profile of glomerular RNA from patients suffering from type II diabetes mellitus, and glomerular RNA from individuals with normal renal function and histology.

Material and Methods

Patients

Cadaveric donor kidneys were obtained from Eurotransplant. These kidneys were unsuitable for transplantation for technical or morphological reasons (Table 1). We used glomeruli from two control kidneys, and from two kidneys from patients with diabetes mellitus type II. Diabetic nephropathy was histologically confirmed by Periodic acid-Schiff (PAS) stained paraffin sections. Pathologic criteria for diabetic nephropathy include glomerular hypertrophy, diffuse mesangial and focal nodular glomerulosclerosis, arteriolar hyalinosis, and focal and segmental glomerulosclerosis, hyaline drops between Bowman's capsule and epithelial cells, and interstitial fibrosis. Nodular glomerulosclerosis, arteriolar hyalinosis are characteristic for diabetic nephropathy, and present in the diabetic kidneys that we have used for this study.

Isolation of glomeruli

Glomeruli were isolated as described earlier (8). In brief, fresh cortical tissue was first pressed with a flattened glass pestle through a 212 µm pore diameter metal sieve and then through a

150µm pore diameter metal sieve. Glomeruli were rinsed from the surface of the 150 µm sieve with ice-cold phosphate buffered saline (PBS), transferred to a tube and pelleted for 1 min at 1200Xg. The supernatant was removed and the glomeruli were frozen at -70°C until RNA isolation. The purity of the glomerular suspension was controlled by light-microscopy and was at least 90%.

RNA isolation

Glomerular RNA was isolated using a combination of two RNA isolation procedures. Glomerular tissue (500mg) was dissolved in 5 ml Trizol® and homogenized with an ultra-turrax (Janke & Kunkel) for 1 min. After adding 1ml chloroform and mixing for 1 min, the suspension was centrifuged at 15,000g for 10 min. The RNA was precipitated with isopropanol. The pellet was air-dried and dissolved in 100 µl MilliQ and further purified with an RNeasy Mini column (QIAGEN GmbH, Germany), according to the instructions of the manufacturer.

Table 1. Characteristics of the patients.

	Control 1	Control 2	Diabetes 1	Diabetes 2
Retinopathy	no	no	yes	yes
Duration of diabetes type 2 (years)	-	-	> 5	>5
Age (years)	29	70	55 years	63 years
Gender	Male	Male	Male	Male
Serum Creatinin (mg/dL)[1]	0.68	unknown	1.14	unknown
Serum Glucose(mg/dL)[1]	133	128	326	unknown
Urine glucose[1]	negative	negative	++	++
Urine Protein[1]	negative	trace	+	+/-
GFR(mL/min)[1]	181	unknown	78	unknown
Perfusion fluid	UW[2]	UW[2]	UW[2]	UW[2]
Cold ischemia time (hours)	32	33	26	32
Dopamine (µg/kg BW/min)	3	0.2 (Norepinephrine)	2	3
Known other drugs	-	-	Insulin	Insulin
Cause of death	ICB[3]	ICB[3]	ICB[3]	ICB[3]
Reason of refusal	Lesion upper arterial pole	Arteriosclerosis	DN	DN
Percentage of sclerotic glomeruli	<1%	<1%	33%	24%
Percentage of interstitial fibrotic area	< 5%	< 5%	25-50%	25-50%

[1] Levels within last 24 hours of donation, [2] University of Wisconsin solution, [3] Intracerebral bleeding.

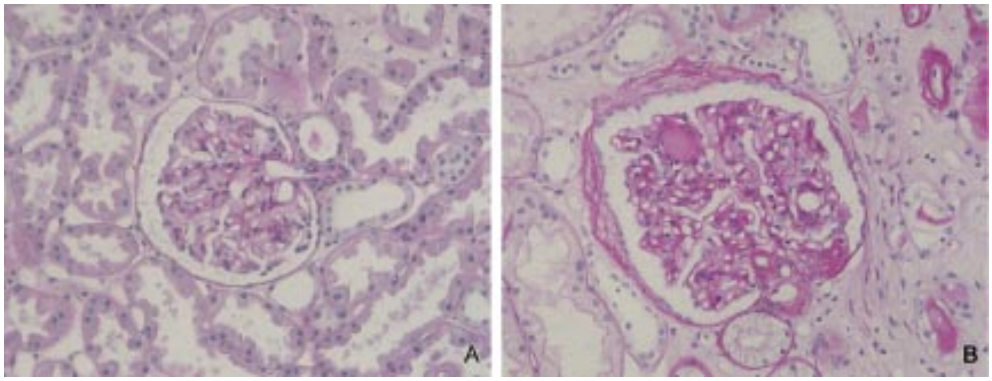

Figure 1. Light microscopic pictures of a glomerulus from a control kidney (A) and a representative glomerulus from a diabetic kidney (B). The diabetic kidneys show glomerular hypertrophy, diffuse mesangial and focal nodular glomerulosclerosis, and arteriolar hyalinosis. (PAS staining, original magnification 200x)

To assess the quality of the RNA, 2 µg of RNA was applied on a 1% agarose-formalin gel. Electrophoresis was performed for 3 h at 50 V. The gel was stained with ethidium bromide.

Microarray hybridization

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 isolated glomeruli of each kidney 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 levels for each gene was multiplied with the normalization factor. Statistics behind 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 determine the inter-assay variation, the labeling procedure and hybridization for one of the controls and one of the diabetic glomerular samples were performed in duplicate. A total of six chips were hybridized, three with control RNA, and three with RNA from diabetic glomeruli.

Confirmation of microarray data by real-time PCR

We performed real-time polymerase chain reaction (PCR) (9), in combination with the Taqman

probe technique, for three genes to confirm the data obtained with the microarray analyses. RNA (1µg) was converted to cDNA using avian myeloblastosis virus (AMV) reverse transcriptase (Roche Applied Science). The transcription levels for nephrin, transforming growth factor-beta (TGF-β), and vascular endothelial growth factor (VEGF) were determined and corrected to a panel of five different housekeeping genes, i.e., glyceraldehyde-phosphate-dehydrogenase (GAPDH), beta-2 microglobulin (B2M), hypoxanthine phosphoribosyl transferase (HPRT), porphobilinogen deaminase (PBGD) and TATA box-binding protein (TBP), as described by Vandesomple et al. (10). The primer and probe sequences are summarized in Table 2. 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 5 different household 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 5 different housekeeping genes was calculated. The relative expression level of VEGF, TGF-ß and nephrin was calculated by dividing the value of the gene by the mean of the different household genes. The relative values were set to one for the controls.

We also measured the relative mRNA levels for VEGF, TGF-ß and nephrin in microdissected glomeruli of 5 renal biopsies from patients with diabetic nephropathy according the method of Specht et al. (11). In brief, 4 µm frozen sections were put on a polyethylene foil coated slide. To microdissect the glomeruli, we used the PALM Laser-MicroBeam System (P.A.L.M., Wolfratshausen, Germany). RNA from the micro-dissected glomeruli was isolated with the TRIzol method as described above. All 5 diabetic patients were suffering diabetes type II for at least 5 years with retinopathy and DN. Renal biopsies of these patients showed glomerular

Table 2. Primer and Taqman® probes sequences.

	Forward primer	Reverse primer	Taqman® probe	Reporter
TGF-β	CCCAGCATCTGCAAAGCTC	GTCAATGTACAGCTGCCGCA	ACACCAACTATTGCTTCAGCTCCACGGA	FAM
VEGF	AAACCCTGAGGGAGGCTCC	TACTTGCAGATGTGACAAGCCG	CCTCCTGCCCGGCTCACCGC	TET
Nephrin	AGGACCGAGTCAGGAACGAAT	CTGTGAAACCTCGGGAATAAGACA	TCAGAGCTCCACGGTCAGCACAACAG	TET
GAPDH	TTCCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG	CCCAGCCTTCTCCATGGTGGTGAA	TET
B2M	TGCCGTGTGAACCATGTGA	CCAAATGCGGCATCTTCAA	TGATGCTGCTTACATGTCTCGATCCCACT	TET
HPRT	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT	CTTGACCATCTTTGGATTATACTGCCTGACCA	TET
PBGD	CTGGTAACGGCAATGCGGCT	GCAGATGGCTCCGATGGTGA	CGAATCACTCTCATCTTTGGGCT	TET
TBP	CACGAACCACGGCACTGATT	TTTTCTTGCTGCCAGTCTGGAC	TGTGCACAGGAGCCAAGAGTGAAGA	FAM

hypertrophy, diffuse mesangial and focal nodular glomerulosclerosis, arteriolar hyalinosis, focal and segmental glomerulosclerosis, and interstitial fibrosis. The relative mRNA levels for VEGF, TGF-ß and nephrin in microdissected diabetic glomeruli were compared to those in glomeruli from 8 control samples, which were described previously (12)

Immunohistochemistry

To validate difference in mRNA for VEGF and nephrin at the protein level, immunohistochemical (IHC)stainings were performed using specific antibodies. For the VEGF staining, 4 µm paraffin sections of the control and diabetic kidneys were cut. After removing the paraffin, the sections were pre-treated with 0.4% pepsin for 20 min at 37°C. For the nephrin staining, we used 3 µm cryostat sections. The slides were washed in PBS and incubated for 2 h at room temperature with the primary antibody, diluted in 1% bovine serum albumin in PBS (rabbit anti-nephrin 1:1000, a generous gift of Dr. Kawachi (12); rabbit anti-VEGF 1:100, Santa Cruz Biotechnology, CA, USA). After washing with PBS, the slides were incubated for 30 min with horseradish peroxidase–conjugated anti-rabbit Envision (DAKO, Glostrup, Denmark). The slides were washed in 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.

Statistics

To determine the reproducibility of the individual microarray analyses within and between groups (i.e. the control group and the diabetic nephropathy group) we calculated coefficients of correlation. Clustering analysis was performed using Spotfire® 7.1 software. We used the Z-score normalization to normalize our data. The normalized value for gene a is calculated as: (a) = (a- mean value of all samples for gene A)/ STD (*A*), where (a) is the normalized value, a is

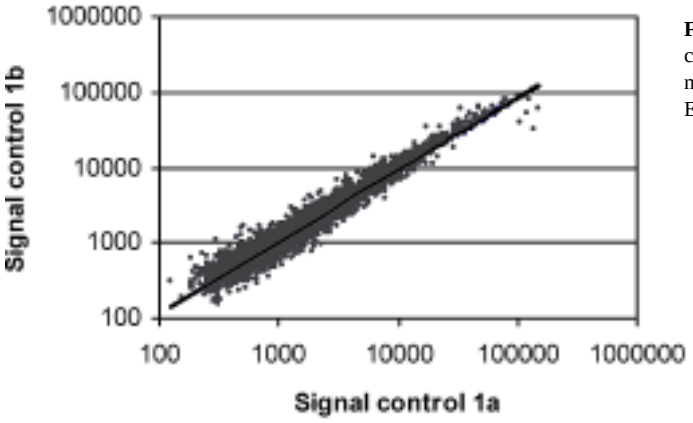

Figure 2. A graph of the correlation between duplicate measurements of a control kidney. Each point represents one gene.

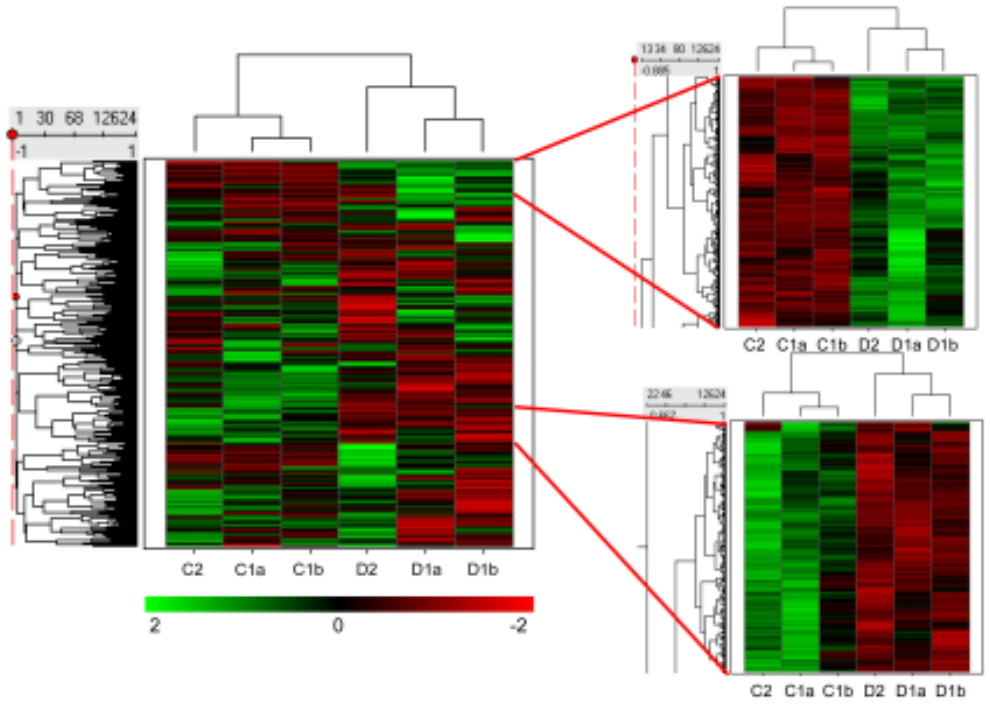

Figure 3. Dendrogram of unsupervised hierarchical clustering on the basis of similarity in gene-expression patterns of the six different arrays. (C1a = control 1, C1b = duplicate of control 1, C2 = control 2, D1a = diabetes 1, D1b = duplicate of diabetes 1, D2 = diabetes 2). The dendrogram showing the degree of relationships of samples. The different colors showed the normalized Z-score for each gene. The normalized Z-score for gene a is calculated as: (a) = (a- mean value of all samples for gene A)/ STD (*A*), where (a) is the normalized value, a is the value of sample a for gene A. Green are upregulated genes and red are downregulated genes.

the value of sample a for gene A. If all values for gene A are identical, then all values for gene A are set to zero. These normalized expression values of the six different arrays were analyzed in an unsupervised fashion using the hierarchical clustering method with complete linkage and correlation. The data was ordered by average value and visualized in a dendrogram.

To identify genes of which expression was altered consistently in the diseased 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 those which were absent in all three control samples and present in all three diabetic samples. We employed multiple pair-wise comparisons between control and disease groups using the OpenStat statistics package. We selected only those genes for which the mRNA level showed an at least 2-fold difference between controls and diabetic samples (students t-test, $p<0.01$).

Gene clustering on basis of Gene Ontology (GO), to identify gene clusters on the basis of gene function, was performed with the MAPPfinder1.0 program (13). MAPPFinder, which can

be downloaded from http://www.genmapp.org, is a program that works in combination with GenMAPP and Gene Ontology to identify global biological trends in gene expression data. MAPPFinder relates microarray data to each term in the (GO) hierarchy, calculating the percentage of genes changed for each GO biological process, cellular component, and molecular function term. Using this percentage and a z- score based on the mean and standard deviation of the hypergeometric distribution, the user can order by GO function with the highest z score. This z-score is calculated as:

$$Zscore = \frac{\left(r - n\frac{R}{N}\right)}{\sqrt{n\left(\frac{R}{N}\right)\left(1-\frac{R}{N}\right)\left(1-\frac{n-1}{N-1}\right)}}$$

where : N = the total number of genes measured, R = the total number of genes meeting the criterion, n = the total number of genes in this specific GO term, and r = the number of genes meeting the criterion in this specific GO term.

Statistical analysis for the real-time data was performed using the one-way analysis of variance (ANOVA), and values of $p<0.01$ were considered to be significant.

Results

Patient characteristics

Characteristics of the donors are summarized in Table 1. Both kidneys with diabetic nephropathy were obtained from patients with a clinical history of type II diabetes for at least 5 years. Gender, cold-ischemia time, the type of perfusion fluid used, and cause of death were similar for the patients. Serum glucose of the control patients was normal, while the glucose levels in the diabetic patients were elevated (up to 18.1 mmol/L). Consistent with a diagnosis of diabetic nephropathy, the urinary protein level in the diabetic patients was increased. The control kidneys showed a normal morphology without histological abnormalities. Both diabetic kidneys showed glomerular hypertrophy, diffuse mesangial and focal nodular glomerulosclerosis in 20-30% of the glomeruli, arteriolar hyalinosis, and focal and segmental glomerulosclerosis. Interstitial fibrosis was seen in 25-50% of the tubulo-interstitial area (Fig. 1).

Gene expression profiles of control and diabetic glomeruli.

From the approximately 12,000 genes displayed on the microchip, 2042 genes gave a positive signal on all three chips after hybridization with the RNA from the kidneys with diabetic nephropathy. In the glomeruli from control kidneys, 4297 genes gave a positive signal in all three samples after hybridization. The correlation coefficient between duplicate control samples

and duplicate diabetic samples were 0.972 and 0.932 respectively. A graph of the correlation between a duplicate of control 1 is shown in Fig. 2. The correlation between controls 1 and 2 and the two different diabetic samples were also high (0.930 and 0.900 respectively). The mean of the correlations between the different control samples and the different diabetic samples was lower (0.731). Unsupervised hierarchical clustering of the expression data as visualized in a dendrogram (Fig 3), shows the same relations between the samples. This dendrogram is based on the similarity between the different samples. By this method the software recognized the highest similarity between duplicate hybridizations, between all three controls, and between all three diabetic samples.

Using the statistics as mentioned in the materials and method we end up with a list of 96 candidate genes that were increased in DN and 519 that were decreased genes. A list of the top fifty of the upregulated genes in the diabetic glomeruli is presented in Table 3 (ratios varying between 2.3 and 4.9 fold). The fifty most down-regulated genes are presented in Table 4 (ratios varying between 6.6 and 22.8 fold). In these lists the unidentified ESTs are not shown. A list of all significantly up- and down regulated genes can be found on: www-onderzoek.lumc.nl/pathology/kidney/diabeticnephropathy/ .

Analysis of the genes, that were either increased or decreased, with Mappfinder was performed to cluster the genes on basis of their GO function. The results are summarized in Table 5. If we look at the results of the decreased genes there is a high z-score for actin cytoskeleton and actin binding GO function and for nucleobase, nucleoside, nucleotide and nucleic acid metabolism. The increased genes are especially related to homeostasis and phosphatases.

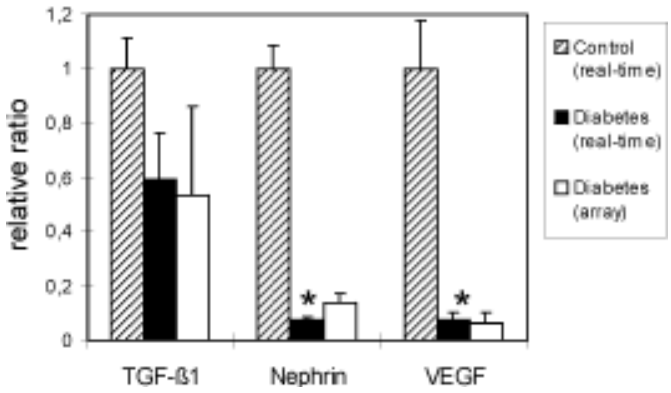

Figure 4. Validation of microarray results for TGFß1, nephrin, and VEGF by real-time PCR. Data have been normalized for a panel of five different housekeeping genes, while the array data are normalized for total chip signals and compared to the control kidneys. (* P<0.001 compared to control (one-way ANOVA)).

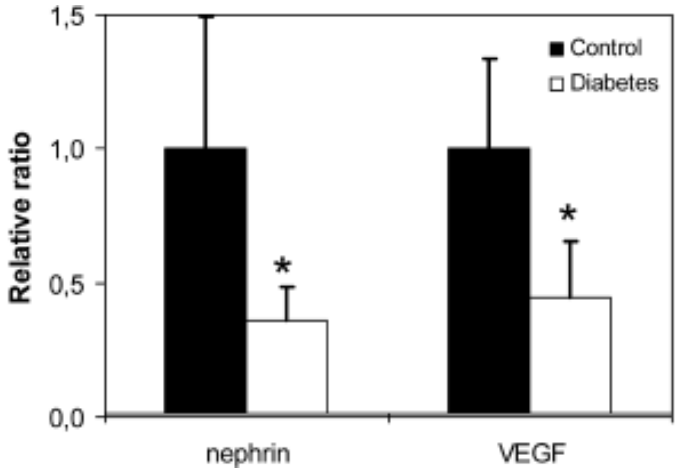

Figure 5. Nephrin and VEGF mRNA levels measured with real-time PCR in an independent group of 5 patients with diabetic nephropathy. Relative levels were compared to a panel of 8 control kidneys (* P<0.05 compared to control (students t-test)).

Confirmation of microarray data by real-time PCR.

To validate the results obtained with microarray, we performed real-time PCR assays for several transcripts. Results of the quantification of mRNA levels for TGF-ß, nephrin, and VEGF are summarized in Fig 4. With microarray and real-time PCR the ratios between TGF-β1 from the controls and diabetic kidneys were found not to be significant (1.87 and 1.82, P=ns). Array analysis showed that nephrin was downregulated (7.3-fold) in DN. Real-time PCR for nephrin also showed a decrease (15.4-fold, P<0.01 compared to the controls) in DN. For VEGF the ratios were 19.5 and 14.2, (P<0.01 compared to the controls) respectively. There was no significant difference between the ratios measured with the microarray and real-time PCR techniques. We also confirmed our data in an independent and larger patient group. The results of these measurements are shown in Fig 5. We found a significant decrease of 2.75 times for nephrin and 2.25 times decrease for VEGF (p<0.05).

Immunohistochemistry

Results for VEGF and nephrin at the RNA level were further investigated at the protein level using IHC. In normal kidneys, VEGF and nephrin showed an intense epithelial staining along the peripheral capillary loops of the glomeruli (Fig 6 A and C). VEGF also showed a weak staining in some tubular epithelial cells. In glomeruli of diabetic kidneys, the staining for both VEGF and nephrin was weaker or absent (Fig 6 B and D).

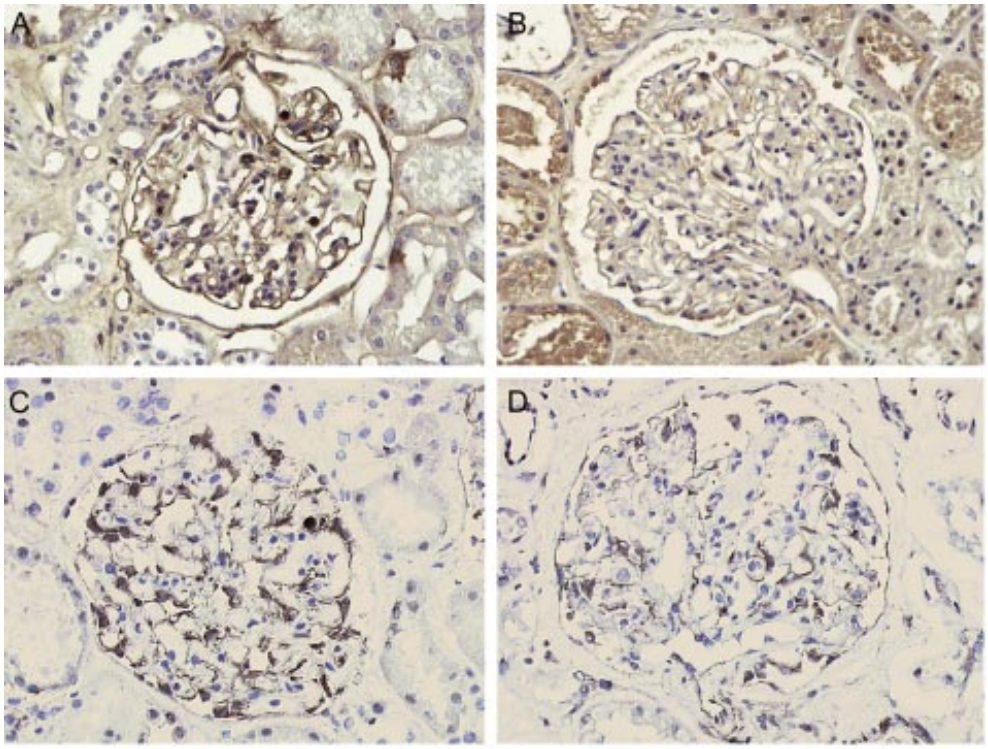

Figure 6. Representative photographs of renal tissue stained for VEGF or nephrin. The upper panel shows VEGF staining on paraffin sections of glomeruli of a control (A) or a diabetic (B) kidney. The control kidney shows abundant VEGF staining in glomerular podocytes. This staining was reduced in the glomerulus of a diabetic kidney. The lower panel shows the nephrin staining on frozen sections of a control (C) or a diabetic (D) kidney. Nephrin was also reduced in the podocytes of the diabetic kidney. Original magnification 200x

Discussion

In this study, we describe gene profiles of control and diabetic glomeruli from human kidneys. RNA was extracted from isolated glomeruli of cadaveric donor kidneys. These kidneys were unsuitable for transplantation due to, non-kidney involved, technical reasons. It is known that these kidneys have been exposed to ischemia, which can alter the gene expression (14). For this reason we compared diabetic kidneys with control kidneys that underwent the same handling prior to the isolation of glomeruli. The isolation was performed on ice and took about 5-10 min for each kidney. From other studies it is known that handling of the glomeruli on ice within 3 hours does not alter the mRNA expression for several pro-fibrotic genes (15). The labeling procedure and hybridization from one of the controls and one of the diabetic glomerular samples were performed in duplicate to calculate the inter-assay variation. The correlation

coefficient was near to 1 indicating that the labeling and hybridization procedure is highly reproducible. The correlation between different control samples and between different diabetic samples was also very high, indicating relatively low heterogeneity within groups. On the other hand the correlation coefficient between control and diabetic samples was lower, reflecting higher heterogeneity between groups. This was also found with the hierarchical clustering analyses (Fig. 3). By unsupervised analysis of the data, the program recognized gene clusters specific for control and diabetic samples based on their correlation. These findings supports the idea that, given the observations that the inter-assay variation and the variation of the gene expression of samples within one group are relatively low, factors such as ischemia, technical procedure, and biological variation probably influence the expression data to only limited extent.

To confirm the data obtained from the microarray, we performed real-time PCR for nephrin, VEGF, and TGF-β1. The relative levels for nephrin and VEGF were significantly decreased in DN compared to controls. No significant differences were observed between the real-time measurements and the microarray results. With both techniques the difference in TGF-β between the controls and diabetic kidneys was found not to be significant. To validate that our findings obtained with cadaveric donor kidneys apply to renal biopsy material, we also measured the mRNA levels of nephrin and VEGF in renal biopsy specimens from 5 patients with diabetic nephropathy and from 8 controls. These patients similarly showed a decrease in message for nephrin and VEGF. To show where the protein was present immunohistochemistry was performed for nephrin and VEGF. We found that VEGF and nephrin in particular were present in the podocytes along the glomerular basement membrane. At the protein level a decrease for these molecules was detected, a finding in line with that at the RNA level.

In the diabetic kidneys more genes were downregulated than there were upregulated compared to controls. This is in accordance with the fact that the number of genes on the chip giving a positive signal after hybridization (present genes) for the diabetic glomeruli was lower than the number of present genes on the chips for the controls (2042 versus 4297). These results are suggestive that downregulation of genes occurs considerably more often in the development of diabetic nephropathy. This idea is supported by the results of Mappfinder. The nucleotide metabolism is in the top 10 of decreased GO functions. There is also a reduction in diabetic nephropathy of the mRNA level of genes that are involved in the formation of the actin skeleton. Downregulation of these pathways in diabetic nephropathy might in part account for the general downregulation seen for many other genes on the chip. Another reason for the difference in the number of present genes between diabetic samples and control samples might be the stringency with which the microarray analyses were performed. We wanted to be sure to include only highly reproducible data in our list of differentially expressed genes. Therefore

Table 3. Top fifty of the most increased genes in all diabetic glomeruli versus controls.

Accession	Ratio	Gene name	Gene function
AI547258	4.9	metallothionein 2A	protects against heavy-metal toxicity
U96078	4.7	hyaluronoglucosaminidase 1	involved in cell proliferation, migration and differentiation
M16941	4.5	major histocompatibility complex, class II, DR	antigen presentation
U02388	4.3	cytochrome P450, subfamily IVF, polypeptide 2	Leukotriene B4 omega-hydroxylase
X85030	3.9	calpain 3	skeletal muscle-specific calcium-dependent cysteine protease
D17793	3.8	aldo-keto reductase family 1	catalyze the conversion of aldehydes and ketones
U19599	3.6	B-cell lymphoma-2-associated X protein	an apoptotic activator
X58288	3.3	protein tyrosine phosphatase, receptor type, M	signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation
D83402	3.2	prostacyclin synthase	vasodilator and inhibitor of platelet aggregation
H94881	3.2	FXYD domain-containing ion transport regulator 2	Gamma subunit of the Na+/K+-ATPase
AB020722	3.0	Rho guanine exchange factor 15	form a complex with G proteins and stimulate Rho-dependent signals.
M25915	3.0	clusterin (complement lysis inhibitor)	plays a role in the terminal complement reaction
J05257	3.0	dipeptidase 1	zinc-dependent metalloprotease
X16832	2.9	cathepsin H	lysosomal cysteine (thiol) proteinase
D13640	2.8	protein phosphatase 1F	inactivates the p21-activated kinase (PAK)
D87002	2.8	immunoglobin light chain	inflammation
AB018258	2.8	Adenosine tri phosphate(ATP)ase, Class V, type 10B	ATPase activity
U95299	2.7	Notch homolog 4 (Drosophila)	has multiple epidermal growth factor (EGF)like, notch, and ankyrin repeats
L48215	2.7	hemoglobin, beta	transports oxygen and carbon dioxide
L11702	2.7	glycosylphosphatidylinositol phospholipase D1	hydrolyzes inositol-PO4 linkage in PtdIns-glycan anchored proteins
U09577	2.7	hyaluronoglucosaminidase 2	lysosomal enzyme
Y07846	2.7	growth arrest-specific 2 like 1	an actin-associated protein expressed at high levels in growth-arrested cells
X64559	2.6	tetranectin	functions in mineralization during osteogenesis
L13720	2.6	growth arrest-specific 6	involved in the stimulation of cell proliferation
M73554	2.6	cyclin D1	alters cell cycle progression
AI762547	2.5	protein phosphatase 3	Ca(2+)-dependent modifier of phosphorylation status
M93311	2.5	metallothionein 3	inhibits cortical neuron survival and neurite formation
J03910	2.5	metallothionein 1G	protect against reactive oxygen species and metals
X58022	2.5	corticotropin releasing hormone-binding protein	inhibits stimulation of pituitary adrenocorticotropic hormone release
D90144	2.5	macrophage inflammtory protein 1-alpha	Small inducible cytokine
X58288	2.5	protein tyrosine phosphatase, receptor type, M	signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation

Table 3. Top fifty of the most increased genes in all diabetic glomeruli versus controls (continued).

Accession	Ratio	Gene name	Gene function
L33930	2.5	CD24 antigen	glycosyl phosphatidylinositol-linked glycoprotein that differentiates and activates granulocytes and B lymphocytes
AB009698	2.5	solute carrier family 22	Renal p-aminohippurate/alpha-ketoglutarate exchanger
AA100961	2.5	PECAM1	transendothelial migration of leukocytes, angiogenesis, and integrin activation
U67733	2.5	phosphodiesterase 2A	hydrolyzes cyclic adenosin monophosphate and cyclic guanine monophosphate
U45973	2.5	kidney enriched inositol phosphatase	may negatively regulate actin cytoskeleton
X07732	2.5	hepsin	transmembrane serine protease
AI017574	2.4	cysteine-rich protein 1	may function as a zinc carrier protein
U03056	2.4	hyaluronoglucosaminidase 1	involved in cell proliferation, migration and differentiation
M13929	2.4	c-myc	Promotor for c-myc
U21931	2.4	fructose-1,6-biphosphatase	fructose-1,6-biphosphatase
L06139	2.4	tyrosine kinase, endothelial (TEK)	critical for endothelial cell-smooth muscle cell communication
S53911	2.4	CD34 antigen	Cell surface antigen expressed on hematopoietic stem cells, and vascular endothelium
AB002438	2.4	Semaphorin 6B	migration
U41518	2.4	aquaporin 1	water channel protein
U11863	2.4	amiloride binding protein 1	deaminates putrescine and histamine
AJ001015	2.4	receptor (calcitonin) activity modifying protein 2	involved in core glycosylation
AF004230	2.3	leukocyte immunoglobulin-like receptor B1	binds cellular and viral major histocompatibility complex (MHC) class I antigens
U40391	2.3	serotonin N-acetyltransferase	enzyme in melatonin synthesis
X65784	2.3	cell matrix adhesion regulator	promotes adhesion of cells to components of the extracellular matrix

Table 4. Top fifty of the most decreased genes in diabetic glomerular samples versus control glomerular samples.

Accession	Ratio	Gene name	Gene function
L76465	22.8	hydroxyprostaglandin dehydrogenase 15	inactivates many prostaglandins
M11810	20.6	2'-5' oligoadenylate synthetase gene	catalyze the synthesis of 2-prime,5-prime oligomers of adenosine
M63978	19.5	vascular endothelial growth factor	mitogen for vascular endothelial cells
M22489	18.3	bone morphogenetic protein 2	member of the TGF-beta family of growth factors
Y16241	15.5	nebulette	binds to actin, tropomyosin, and alpha-actinin
AF009314	15.1	TUA8 Cri-du-chat region	unknown
AF042377	15.0	mannose 4,6-dehydratase	plays a role in the synthesis of fucosylated oligosaccharides

Table 4. Top fifty of the most decreased genes in diabetic glomerular samples versus control glomerular samples (continued).

Accession	Ratio	Gene name	Gene function
Z48541	14.7	glomerular epithelium protein 1	receptor-type protein tyrosine phosphatase
AI207842	14.3	prostaglandin D2 synthase	catalyzes synthesis of prostaglandin D
J03779	14.0	membrane metallo-endopeptidase	inactivates several peptide hormones including glucagon
L13698	13.6	growth arrest-specific 1	plays a role in growth suppression
L12468	13.5	aminopeptidase A	glycosylated zinc-dependent metalloprotease
X59065	12.7	fibroblast growth factor 1	potent mitogen for a variety of cell types
U49392	12.5	Allograft inflammatory factor 1	cytokine inducible protein associated with vascular injury
Y07593	12.2	coxsackie virus and adenovirus receptor	receptor for coxsackievirus and adenovirus
AB014524	11.9	SLAC2-B	unknown
D78014	11.9	dihydropyrimidinase-like 3	mediate signals involved in axonal outgrowth
AF078096	11.3	forkhead/winged helix-like transcription factor 7	transcription factor
X81053	11.2	collagen, type IV, alpha 4	extracellular matrix protein that forms basement membranes
X04371	10.9	2',5'-oligoadenylate synthetase 1	catalyze the synthesis of 2-prime,5-prime oligomers of adenosine
AB029000	10.5	sulfatase FP	sulfatase
U17034	10.4	phospholipase A2 receptor 1	Secretory phospholipases A2 receptor
L28997	9.7	adenosin-di-phosphate-ribosylation factor-like 1	stimulate phospholipase D
U65887	9.4	cytosin-di-phosphate (CDP)-diacylglycerol synthase	converts phosphatidic acid to CDP-diacylglycerol
S37730	9.3	insulin-like growth factor binding protein-2	binds to and modulates insulin-like growth factor activity
X73608	9.2	sparc/osteonectin(testican)	function may be related to protease inhibition.
U24152	9.1	p21/Cdc42/Rac1-activated kinase 1	regulates cell motility and morphology.
X14034	9.0	phospholipase C, gamma 2	hydrolyzes phosphatidyl inositol
M22489	8.6	bone morphogenetic protein 2	signals through receptor serine/threonine kinases
AF047419	8.4	epicardin, podocyte-expressed 1	transcription factor
M97935	8.4	Signal Transducer and Activator of Transcription 1	transcription factor
L17418	8.3	complement receptor type 1	binds complement
AB014605	8.2	atrophin-1 interacting protein 1	interact with atrophin-1
X74819	8.2	troponin T2	the tropomyosin-binding subunit of troponin
M24594	8.1	interferon-induced protein	unkown
U42360	7.9	Putative prostate cancer tumor suppressor	Putative integral membrane tumor suppressor protein
J02931	7.8	coagulation factor III	initiates the coagulation protease cascade assembly and propagation
M97936	7.7	IFN-stimulated gene factor-3 (ISGF-3)	transcription factor
L25124	7.6	prostaglandin E receptor 4	receptor that signals through a stimulatory G-protein
AF022375	7.5	vascular endothelial growth factor	induces endothelial cell proliferation and vascular permeability
U50534	7.5	breast cancer 2 (BRCA 2)	involved in DNA repair processes

Table 4. Top fifty of the most decreased genes in diabetic glomerular samples versus control glomerular samples (continued).

Accession	Ratio	Gene name	Gene function
AB022918	7.2	alpha2,3-sialyltransferase	plays a role in synthesis of sialyl-paragloboside
AB006746	7.1	phospholipid scramblase 1	plays a role in the EGF-induced metabolic or mitogenic response.
U18934	7.0	protein tyrosine kinase	receptor protein tyrosine kinase
AJ001381	7.0	myosin IB	Member of the myosin family of motor ATPases
D17517	7.0	TYRO3 protein tyrosine kinase	Receptor protein tyrosine kinase
Y08374	7.0	cartilage GP-39	associated with monocyte to macrophage maturation
M62424	6.9	coagulation factor II receptor	involved in platelet activation
J02611	6.7	apolipoprotein D	component of high density lipoprotein
AI401567	6.6	glutamate receptor	ligand-gated ion channel selectively permeable to sodium and calcium

only those genes, which were present in all three diabetic arrays, were included. There is more heterogeneity in gene expression patterns among diabetic samples than among control samples. This would mean that, due to this difference in heterogeneity, the chance that a certain gene is positive on all three chips in the diabetic group is lower than the same gene being present in all three chips of the normal samples.

One of the major clinical problems in patients with diabetes is the presence of vascular abnormalities, such as increased endothelial permeability to macromolecules and endothelial proliferation (16). Considerable research has focused on the pathogenesis of endothelial dysfunction, but the exact mechanisms have remained unclear. VEGF is one of the most important factors in endothelial repair and angiogenesis. It has recently been shown that subtotally nephrectomized rats show a reduction of VEGF mRNA in the kidney (17). Treatment of these rats with angiotensin converting enzyme inhibitors leads to normalization of both glomerular VEGF mRNA levels and capillary endothelial cell density. In animal models for diabetic nephropathy, an increase of VEGF was found in diseased renal tissue (18). In contrast, in human renal biopsies with diabetic nephropathy a decrease of VEGF at both the protein and the mRNA level was shown (19). The notion that VEGF mRNA was found to be decreased in human DN is supported by our observations (20). Another gene for which expression was significantly decreased in the diabetic glomeruli is fibroblast growth factor 1 (FGF1). This protein functions as a modifier of endothelial cell migration and proliferation, and an angiogenic factor, and it can protect the kidney against ischemia-reperfusion injury (21). The expression of PECAM-1, a molecule that is involved in angiogenesis and leukocyte trafficking, was increased in the diabetic kidneys.

Accumulation of extracellular matrix (ECM) proteins has been found in animal models and in biopsies from patients

Table 5. Top 10 of the Mappfinder results based on gene ontology (GO) function ranked on basis of highest z-Score.

Mappfinder results based on decreased genes		
GO ID	GO Name	z-Score
7242	intracellular signaling cascade	2.14
5515	protein binding	2.11
15629	actin cytoskeleton	2.07
4	biological_process unknown	1.97
6886	intracellular protein transport	1.95
5488	binding	1.89
8285	negative regulation of cell proliferation	1.83
3779	actin binding	1.83
3677	DNA binding	1.81
6139	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	1.78
Mappfinder results based on increased genes		
GO ID	GO Name	z-Score
19725	homeostasis	4.81
30005	di-, tri-valent inorganic cation homeostasis	4.30
16302	phosphatase	4.25
16791	phosphoric monoester hydrolase	3.84
30006	heavy metal ion homeostasis	3.72
5505	heavy metal binding	3.19
16788	hydrolase, acting on ester bonds	3.13
4437	inositol/phosphatidylinositol phosphatase	3.03
7218	neuropeptide signaling pathway	3.03
19730	antimicrobial humoral response	3.03

with diabetic nephropathy (22). Expansion of the ECM can be the result of a disturbed balance between ECM synthesis and ECM degradation, or a combination of these mechanisms. Of note, we found an increase of message for metargidin, a disintegrin metalloproteinase (23), and a decrease of message for collagen α4(IV), a major structural component of the GBM. In a previous study, an increase for overall collagen type IV protein was observed in glomeruli from patients with diabetic nephropathy (3). In animal cell cultures under high glucose levels an increase in collagen type IV mRNA was mainly found for the alpha 1, alpha 3, and alpha 5 chains (24). In this study we did not find a change in the mRNA level for TGF-β. In the literature the role of TGF-β. has been described in several animal models (summarized in (25)) and a small increase of the mRNA level in human glomeruli have been reported (26,27). A reason of the opposing result for TGF-β between previous studies and our study might be that this molecule was studied in different stages of the disease. Alternatively, the mRNA levels for TGF-β we described in our study might not reflect the level or activity of the corresponding protein. An increase of active TGF-β can also be explained by increased translation, or increased activation of latent TGF-β. A decrease of the natural inhibitors can also increase the bioactivity of TGF-β.

Recently it has also been shown that high-glucose can induce fibronectin and collagen type III expression in renal fibroblasts independent of TGF-β1(28). The growth factor BMP-2, the growth factor inhibitor syndecan-2, and the growth factor receptor insulin-like growth factor binding protein-2 (IGFBP-2) were all decreased in DN. These components are known to play a role in ECM remodeling (29-31).

The diabetic kidneys analyzed in this study morphologically showed glomerular hypertrophy and proliferation, a common event seen in diabetic nephropathy (32). With respect to proliferating cells in diabetic glomeruli, expression profiling of these glomeruli as reported here shows many genes that play an important role in cell cycle regulation. In kidneys with DN, we saw an increase of hyaluronoglucosaminidase 1 and a decrease in BMP-2 and growth arrest-specific 1 protein, all suggestive for increased proliferation. It has recently shown that treatment of streptozotocin induced diabetic rats with BMP-7 preserves the GFR, reduces the proteinuria and prevents glomerulosclerosis (33). For breast cancer 2 (BRCA-2), nedcin, and the cytokines FGF-1 and VEGF, a role in cell cycle control has been described (34-36).

The pathogenesis of albuminuria, one of the earliest clinical signs of diabetic nephropathy, has not been fully clarified. It is generally assumed that the filtration apparatus of the glomerular capillary wall is of central importance in this process. It has been shown that the slit diaphragm located between the foot processes of the podocytes plays a crucial role in the filtration of macromolecules (37). The expression of nephrin, a transmembrane protein that localizes in the slit pore of the glomerular epithelial cells, was found to be decreased in diabetic glomeruli in our study. This observation is in agreement with the reduction of glomerular nephrin gene expression and with the increase in albuminuria at a later stage of the disease both in human diabetic nephropathy (38,39) and in diabetic and hypertensive rats (40). The transcription of podocalyxin, a protein expressed in the slit pore, is regulated by the transcription factor Wilms tumor 1 (WT1)(not present in the top 50, but 6.9-fold decreased) (41). Downregulation of this transcription factor may lead to a lack of podocalyxin. These findings support the hypothesis that slit pore-associated proteins play a role in the development of proteinuria.

In conclusion, we found that in diabetic nephropathy more genes were downregulated than there were upregulated, compared to controls, which might be explained for a large part by a decreased nucleotide metabolism. We also found a disturbed cytoskeleton formation in diabetic nephropathy. Many other tissue repair related genes as BMP-2, FGF-1, insulin-like growth factor binding protein-2 and CTGF were downregulated in diabetic nephropathy, all suggestive for a reduced tissue repair capacity. On top of that, message for VEGF was decreased in DN compared to controls. This finding for VEGF was validated at the protein level, and additionally, tissue VEGF levels were found to be decreased in an independent group of diabetic patients. These findings suggest that the progression of diabetic nephropathy might at least in

part be a result of a diminished repair mechanism in the endothelium of the capillaries.

The results described in this study underscore the potential of gene chip technology as a methodology for unraveling the complexities of the renal response to diabetes mellitus. This powerful technique allows simultaneously analysis of the expression profile of thousands of genes. We discussed several genes differentially expressed between array data sets, which are functionally related to vascular damage, mesangial matrix expansion, proliferation, and proteinuria, events seen in diabetic nephropathy. Further elucidation of the functional involvement of these genes by studies in larger patients groups and time course experiments will lead to an even better understanding of the processes leading to diabetic nephrosclerosis.

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(Colour picture from page 125)

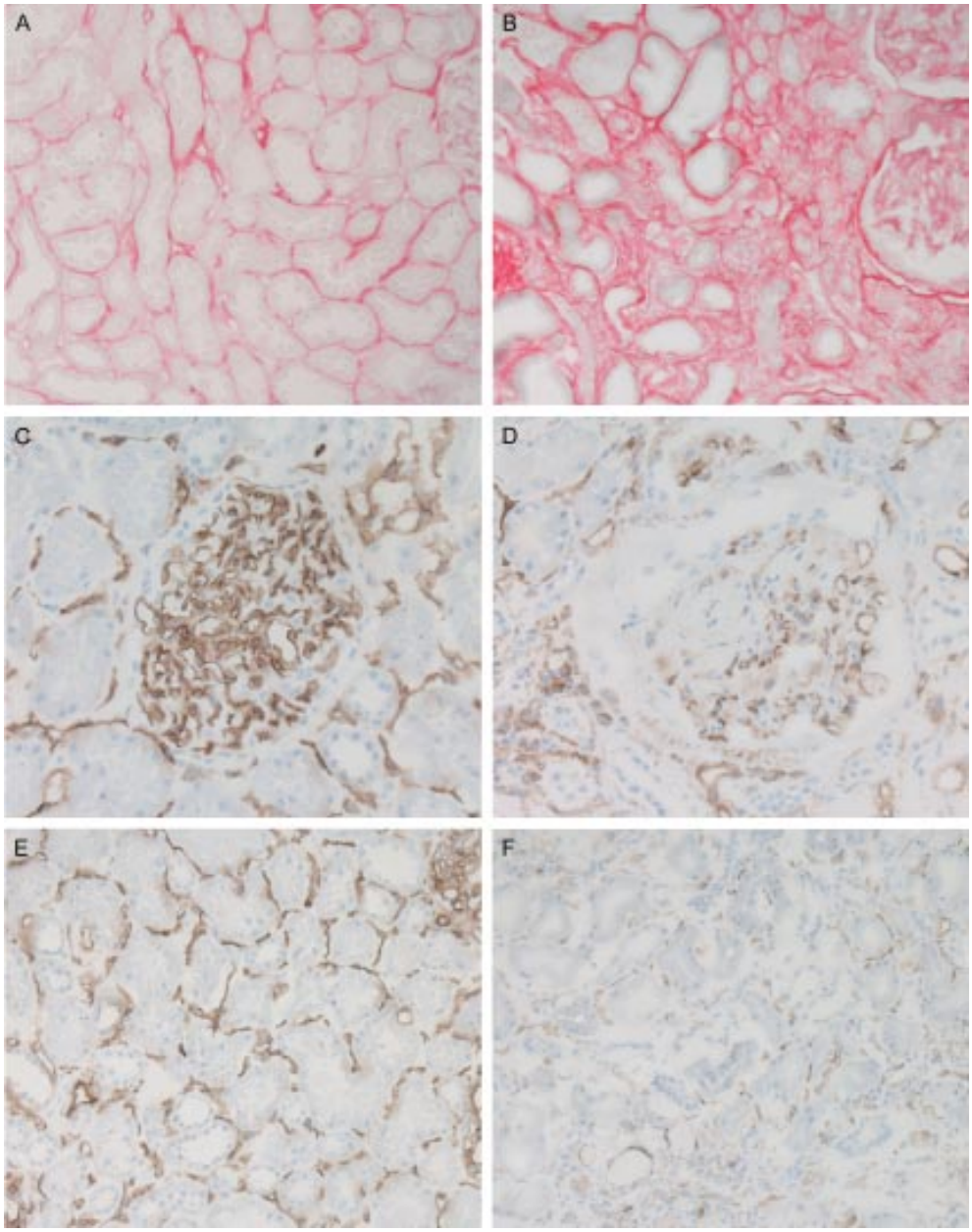

Figure 4. Sirius Red and CD31 staining in the biopsies**.** A and B are representative illustrations of the Sirius Red staining in a control patient and in a patient with DN, respectively. C-F are representative pictures of CD31 staining: glomerulus of a control patient (C), glomerulus of a patient with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