

Enhanced Expression of ANGPTL2 in the Microvascular Lesions of Diabetic Glomerulopathy

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Key Words

ANGPTL2 expression · Diabetic nephropathy · Microvascular complications

Abstract

Background: Diabetic nephropathy (DN) is one of the most important microvascular complications of diabetes mellitus. However, the underlying mechanisms remain unclear. We studied the expression characteristics of angiopoietin-like 2 (ANGPTL2), a novel DN-associated growth factor identified in our previous gene chip screening. **Methods:** Glomeruli were microdissected from renal biopsies from 24 patients with DN and 8 donor controls. The expression of ANGPTL2 was assessed by RT-PCR and immunohistochemistry, and then correlated with clinical and pathological indices of glomerular injury. **Results:** Consistent with the results of the gene chip experiment, abundant expression of ANGPTL2 was found more frequently in diabetic glomeruli as compared to donor controls (95 vs. 38%, $\chi^2 = 15.9$, $p < 0.01$). ANGPTL2 mRNA upregulation was more prominent in glomeruli with less microaneurysm (22 vs. 66%, $p < 0.05$), inflammatory influx (6 vs. 50%, $p < 0.05$) or endothelial foam cell formation (11 vs. 53%, $p < 0.05$). Immunostaining revealed an upregulation of ANGPTL2 protein in hypertrophied diabetic glomeruli, mainly distributed in podocytes, which were sup-

posed to be the origin of ANGPTL2. **Conclusion:** The upregulation of ANGPTL2 in diabetic glomerulopathy shows a close relationship to abnormal microvasculature and endothelial inflammation. ANGPTL2 may play an important role in the pathogenesis of diabetic glomerulopathy.

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Introduction

Diabetic nephropathy (DN) is a complication in 30–40% of patients with type II diabetes and is the most common pathological disorder predisposing to end-stage renal disease. In the pathogenesis of DN, endothelial dysfunction and microvascular disturbances play central roles [1–3]. The angiopoietin family of endothelial growth factors has been found to participate in this course, and a changing balance of these factors contributes to glomerular vascularity [4–8].

Angiopoietins are a group of angiogenesis-associated vascular endothelial growth factors. ANGPTL2 (angiopoietin-like 2) is a newly recognized angiopoietin homologue firstly isolated from human heart cDNA by homologous PCR using angiopoietin-1-specific primers. In vitro studies reveal that ANGPTL2 can induce sprouting of endothelial cells in an autocrine or paracrine manner [9], thus indicating its role in angiogenesis. Our glomerular gene expression profile of DN revealed a significant upregulation of ANGPTL2 (>2-fold) in glomeruli of patients with DN [10]. To exclude the possible bias brought

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about by individual diversity of the patients and to validate the expression characteristics of this gene, we chose to collect a large sample number of renal biopsies from both DN patients and normal controls who were not included in the original gene chip experiment. In addition, correlations between ANGPTL2 expression and phenotype indices of the enrolled patients were analyzed in order to shed light on the possible function of ANGPTL2.

Methods

Patients

From our institution, 24 in-house patients with type II diabetes mellitus and different degrees of albuminuria/renal insufficiency were enrolled in our study; mean ages were 53 ± 10 years, with a male-to-female ratio of 1.1:1. The criterion for the diagnosis of diabetes mellitus was based on recommendations made by the American Diabetes Association in 1997. Biopsies presented with glomerular changes characteristic of the diabetic state, including enlarged glomeruli and increased mesangial matrix with/without Kimmelstiel-Wilson nodules, excluding any other possible complicated primary/secondary glomerulopathy. All patients accepted renal biopsy from September 2003 to February 2005. Redundant biopsy samples were subjected to glomerulus dissection and molecular analysis on the condition that sufficient samples for routine histological, immunological and ultrastructural examinations were collected. All the patients were fully informed about the procedure, and consents were obtained. Biopsy samples from 8 donor kidneys of the same period in our transplantation center were collected and served as normal controls.

Materials and Reagents

Monoclonal anti-human ANGPTL2 and monoclonal anti-human CD31 were bought from R&D Systems. Monoclonal anti-human CD68, FITC-conjugated goat anti-mouse IgG and reagents for 4-layer peroxidase-antiperoxidase immunohistochemical detection were from Dakocytomation. Type IV collagenase was a product of Sigma Inc. Trizol and RNase-free glycogen were from Gibco Inc. A reverse transcription system was bought from Promega Inc.

Glomerulus Microdissection and RNA Extraction

Fresh renal biopsy segments (cortex, $1 \times 1 \times 2$ mm) were incubated in 1 mg/ml type IV collagenase in PBS at 37°C for 20 min. Glomeruli were separated in iced PBS in a dissection dish and rinsed of debris. From each sample, 5 glomeruli were captured with a 20- μl tip and transferred into 400 μl Trizol containing 0.25 $\mu\text{g}/\mu\text{l}$ RNase-free glycogen. Total RNA was extracted using Trizol per manufacturer's instructions and then precipitated with glycogen and stored at -80°C until further use.

Assessment of ANGPTL2 mRNA Levels in Human DN by RT-PCR

RNA was subjected to reverse transcription with oligo-dT primers and reverse transcriptase. The reverse transcription system consisted of 2 μl of $10 \times$ reverse transcription buffer, 4 μl of 25 mM Mg^{2+} , 2 μl of 10 mM dNTP, 0.5 μg of oligo-dT, 0.5 μg of

RNasin and 0.7 μl of avian myeloblastosis virus buffer. The system underwent the following program: 42°C for 1 h, 95°C for 3 min, 4°C for 5 min. cDNA production was added to a 20- μl PCR mixture which included 2 μl of $10 \times$ buffer, 2 μl of 25 mM Mg^{2+} , 1 μl of 10 mM dNTP, 1 μl of 10 μM specific primer and 0.5 units of Taq polymerase. The PCR program was optimized and performed as follows: denaturation at 95°C for 5 min, followed by 35 cycles of amplification (94°C for 30 s, 60°C for 30 s and 72°C for 30 s). The amount of PCR product was normalized with β -actin to determine the relative expression ratio for ANGPTL2 mRNA. The following oligonucleotide primers were used: ANGPTL2 sense, 5'-CGG CAT TGT GAG CGA GGT GAA G-3'; ANGPTL2 antisense, 5'-TGC GGT TGT AGG TGG GTG GTT G-3'; β -actin sense, 5'-GAA GAG CTA CGA GCT GCC-3'; β -actin antisense, 5'-TGA TCC ACA TCT GCT GGA-3'. PCR products were sequenced and confirmed by BLAST at <http://www.ncbi.nlm.nih.gov/blast>.

Immunohistochemistry

Since the tissue for immunohistological detection was precious, we chose formalin-fixed, paraffin-embedded tissue sections of renal biopsies from another 4 DN patients and 2 donor kidneys for immunohistochemical studies. Two-micrometer sections were dewaxed and rehydrated gradually through graded alcohols. Endogenous peroxidase was quenched with 3% H_2O_2 for 30 min, and antigen unmasking was performed by heat treatment at 95°C in 10 mM sodium citrate buffer (pH 6.0) for 10 min. Then the sections were blocked in 10% fetal calf serum and incubated with 1st antibodies (monoclonal anti-human ANGPTL2, dilution 1:100; or monoclonal anti-human CD68, dilution 1:100) overnight at 4°C , followed by 4-layer peroxidase-antiperoxidase immunohistochemical staining and developed with diaminobenzidine. PBS was used instead of the 1st antibodies as a negative control. CD68 immunostaining was performed to mark the infiltrated monocytes. Immunofluorescent staining of CD31 was used to represent endothelial distribution and capillary density of the glomerular tuft. The rehydrated slides were in turn incubated with monoclonal anti-human CD31 (dilution 1:100) at 4°C overnight and FITC-conjugated goat anti-mouse IgG (1:50) at room temperature for 40 min. After the sections had been washed and mounted, the fluorescent signal was captured under Olympus fluorescent microscopy (488 nm).

Clinical Characteristics

Clinical characteristics were studied in 24 enrolled patients with type II diabetes mellitus and biopsy-proven DN. The patients were classified according to the clinical stage of the disease: stage 1 = microalbuminuria, urinary albumin excretion 20–200 $\mu\text{g}/\text{min}$; stage 2 = overt nephropathy, urinary albumin excretion $>200 \mu\text{g}/\text{min}$ or persistent proteinuria exceeding 0.5 g/24 h, with hypertension and declining glomerular filtration rate; stage 3 = end-stage renal disease. The prevalence of renal failure (serum creatinine $>1.4 \text{ mg}/\text{dl}$), hypertension (blood pressure $>140/90 \text{ mm Hg}$) and retinopathy was also investigated.

Histological Indices

Renal biopsy samples were routinely fixed, dehydrated and embedded in paraffin. Sections (2 μm) were stained with periodic acid-Schiff for light-microscopic observation. Morphological characteristics, including enlargement of glomeruli, thickness

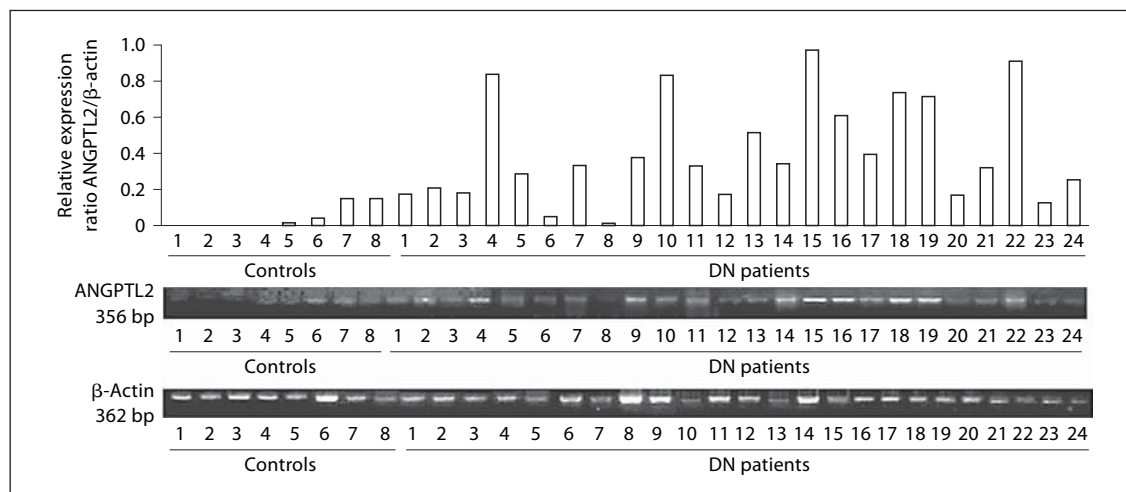


Fig. 1. Gel electrophoresis of ANGPTL2 mRNA expression in glomeruli from renal biopsy samples of 24 patients with DN and 8 donor controls by RT-PCR. The transcriptional level of ANGPTL2 was normalized by that of β -actin.

of basement membrane, expansion of the mesangial matrix (mild, moderate or severe), formation of Kimmelstiel-Wilson nodules, microangioma and endothelial lesions (including leukocyte influx, endothelial proliferation and degeneration) were analyzed. A pathologist unaware of the experimental protocol was in charge of the glomerular histological analysis.

Statistical Analysis

SPSS11.0 software was used to perform the statistical analysis. The χ^2 test was used to identify significant differences between groups. A level of $p < 0.05$ was considered as statistically significant.

Results

Validation of Enhanced Expression of ANGPTL2 mRNA in Patients with DN

The mRNA level of ANGPTL2 was analyzed by semi-quantitative RT-PCR in 24 patients with DN and 8 donor controls. ANGPTL2 mRNA was detected in 23 (95%) out of 24 DN samples and in 3 (38%) out of 8 donor samples. Compared to normal controls, ANGPTL2 was significantly more frequently expressed in diabetic nephropathy ($\chi^2 = 11.62$, $p < 0.01$, fig. 1). This result is in accordance with the preliminary results from our original gene chip experiment. The relative transcript ratio for ANGPTL2 mRNA was calculated for the 32 samples, and receiver-operating characteristic curve analysis of these data was performed to determine a cutoff value for the relative transcript levels of ANGPTL2 that discriminated

DN and non-DN cases. In our experiment, a cutoff value of 0.27 might discriminate a DN from a non-DN individual with a specificity of 88.9% and a sensitivity of 91.7%. A cutoff value of 0.50 might discriminate a DN from a non-DN individual with a specificity of 100% and a sensitivity of 66.7% (fig. 2). The 24 patients were divided into 2 groups, according to their relative transcript ratios: patients with pronounced upregulation of ANGPTL2 (ratio >2 -fold, $n = 9$) and those with moderate transcription (ratio >2 -fold, $n = 15$).

Expression and Distribution of ANGPTL2 Protein in Glomeruli

As detected by immunohistochemistry, enhanced expression of ANGPTL2 protein was found in glomeruli of all the 4 samples from DN patients, while it was almost undetectable in both control kidneys (fig. 3). The increased ANGPTL2 protein was mainly distributed in a podocyte-like pattern in hypertrophied glomeruli of DN patients, suggesting that podocytes may be a major origin of this factor.

Correlations between ANGPTL2 mRNA Expression and Clinical Characteristics

Patients with upregulated ANGPTL2 expression (ratio >2 -fold) have a relatively higher prevalence of retinopathy, hypertension and renal insufficiency (table 1). These 3 characteristics have been considered prognostic indicators for renal survival, but our data did not show

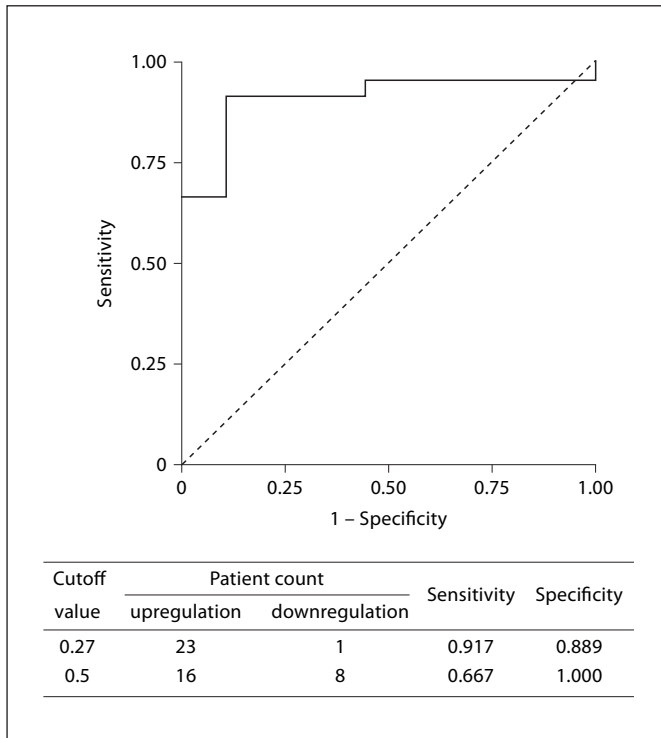


Fig. 2. Receiver-operating characteristic analysis of ANGPTL2 mRNA from DN patients and controls. The table shows cutoff values for ANGPTL2 transcripts that discriminate DN patients and normal controls with high sensitivity and specificity.

significant correlations between gene expression level and these clinical indices ($p > 0.05$). In addition, our data indicated no ANGPTL2 transcription differences in samples of variant clinical stages.

Correlations between ANGPTL2 Expression and Pathological Indices

Arteriolar hyalinization and thickness of basement membranes were ubiquitous changes observed in all 24 enrolled patients, irrespective of ANGPTL2 mRNA levels, and there was no significant difference between nodular lesions (13 cases) and diffuse mesangial proliferation (11 cases). In patients with pronounced ANGPTL2 upregulation (ratio >2 -fold), the emergence of severe mesangial matrix expansion and exudative lesions (fibrin cap and capsular drop) were relatively, but not significantly, higher (75 vs. 63%, 81 vs. 50%, respectively; $p > 0.05$). However, ANGPTL2 transcription was prominent in glomeruli with less leukocyte influx (6 vs. 50%, $\chi^2 = 6.19$, $p = 0.03$), microaneurysm formation (22 vs. 66%, $\chi^2 = 4.44$, $p = 0.04$) and endothelial foam cell degenera-

Table 1. Correlations between ANGPTL2 transcription and patient phenotype

Phenotype of the patients	ANGPTL2 mRNA expression		p
	<2 -fold	>2 -fold	
Age			
<50 years	5 (62)	6 (38)	0.34
>50 years	3 (38)	10 (62)	
Clinical stage			
Microalbuminuria	1 (11)	3 (20)	0.84
Overt nephropathy	5 (56)	8 (53)	
ESRD	2 (22)	5 (33)	
Clinical manifestations			
Retinopathy	6 (66)	12 (80)	0.39
Hypertension	7 (78)	14 (93)	0.27
Renal insufficiency	2 (22)	6 (40)	0.35
Glomerular lesion			
Nodule formation	5 (56)	8 (53)	0.33
Mesangial expansion	5 (56)	12 (80)	0.45
Microvascular/endothelial lesions			
Microaneurysm	6 (66)	3 (22)	0.04*
Leukocyte influx	4 (50)	1 (6)	0.03*
Foam cell formation	8 (53)	2 (11)	0.04*

Figures in parentheses are percentages. Asterisks indicate statistically significant differences. ESRD = End-stage renal disease.

tion (11 vs. 53%, $\chi^2 = 4.28$, $p = 0.04$, table 1, fig. 4) as examined by light microscopy. Figure 3 shows the distribution and expression intensity of ANGPTL2 protein, in correlation with the capillary density represented by CD31 and the inflammatory infiltration marked by CD68. We found that in DN1 and DN2, glomeruli with a relatively lower level of ANGPTL2 expression showed an expanded capillary area and infiltration of monocytes. However, in DN3 and DN4, glomeruli with prominent ANGPTL2 expression presented mainly mesangial proliferation in the absence of inflammatory influx.

Discussion

Our gene chip experiment identified 182 differentially expressed genes in diabetic glomeruli, which is a condition that may participate in the development of diabetic glomerulopathy. Among them are candidate genes that associate with endothelial functions and angiogenesis. Vascular endothelial growth factor was one of these genes

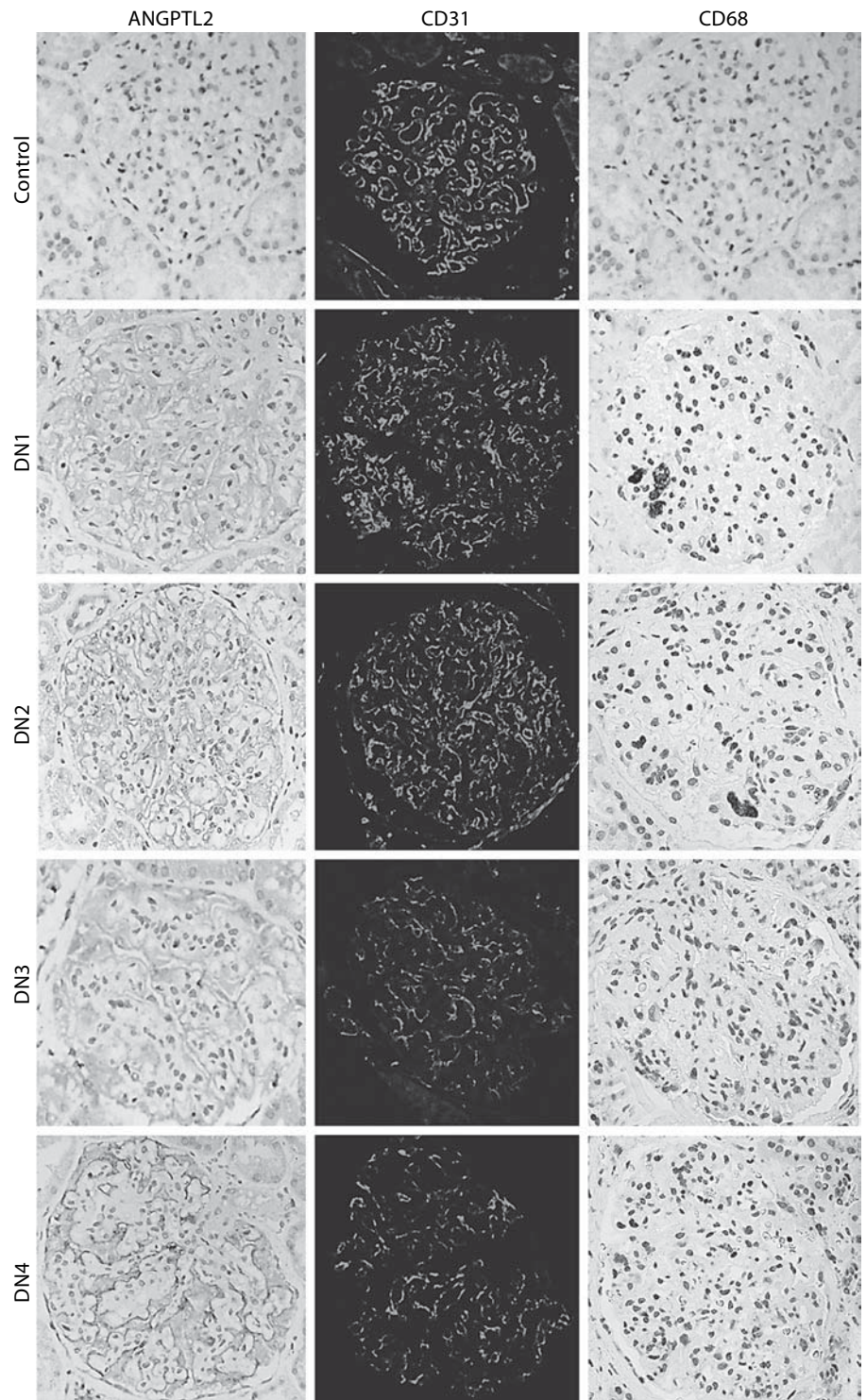


Fig. 3. Immunohistochemical staining of ANGPTL2 in diabetic glomeruli. Tissue slides of renal biopsies from 4 DN patients (DN1, DN2, DN3, DN4) and normal controls were subjected to immunostaining of ANGPTL2, CD31 and CD68. CD31 was used to trace capillaries, and CD68 was used to identify infiltrated monocytes.

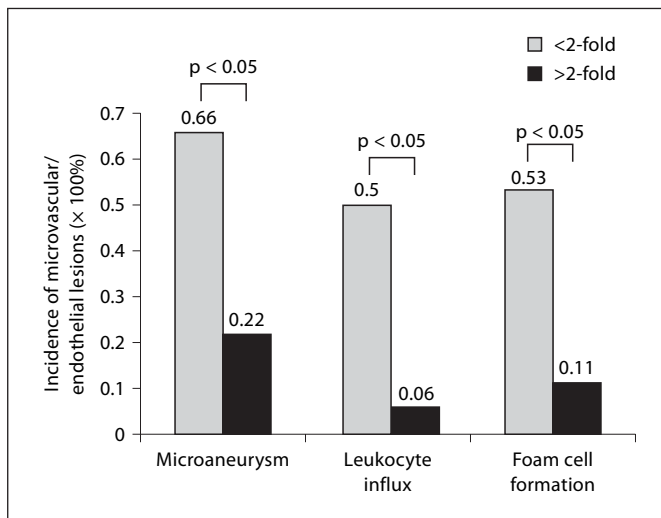


Fig. 4. The 24 patients were divided into 2 groups according to their relative transcript ratios: patients with pronounced upregulation of ANGPTL2 (ratio >2-fold, n = 15) and those with moderate transcription (ratio <2-fold, n = 9). ANGPTL2 transcription was prominent in glomeruli with less leukocyte influx, microaneurysm formation and endothelial foam cell degeneration (6 vs. 50%, 22 vs. 66%, 11 vs. 53%).

and its enhanced expression in diabetic glomeruli was validated in our later work [11]. ANGPTL2 was another angiogenesis-associated vascular growth factor identified in our gene chip analysis. It was significantly enhanced (>2-fold) in DN compared to controls. In the present study, ANGPTL2 mRNA was detected in 95% of the diabetic glomerulus samples and in 38% controls, in accordance with what we learned from our gene chip screening. Immunohistochemical analysis illustrates that ANGPTL2 is a podocyte-derived growth factor. In addition, the upregulated ANGPTL2 protein was detected in the diabetic glomeruli from all the DN patients but absent in normal glomeruli of both control kidneys, which further demonstrates that ANGPTL2 participates in the development of diabetic glomerulopathy.

Our data show that the upregulation of ANGPTL2 mRNA is in negative correlation with the occurrence of glomerular microaneurysm. As a feature of diabetic microvascular lesions, microaneurysm results from glomerular hypertrophy and disarrangement of microvessels by hemodynamic changes, in which the endothelial cells lose their scaffold for growth with the destruction of the mesangium structure [12, 13]. As shown by immunostaining, in hypertrophied glomeruli with increased capillary intensity, with the prominent endothelial prolifera-

tion characterized by CD31, the ANGPTL2 expression was relatively lower. Although we cannot define an exact association between the extent of endothelial abnormality and ANGPTL2 upregulation, we propose that ANGPTL2 may act as an antagonist of microvascular reconstruction and may play an important role in directing the integrity of the glomerular capillary.

DN and diabetic retinopathy are both microvascular complications of diabetes, and morphological and pathophysiological research has revealed many common features between them [14]. Adhesion of leukocytes to the retinal vasculature has been reported as a critical early pathology in diabetic retinopathy: leukocytes adhering to the retinal vasculature may trigger endothelial cell injury and blood-retina barrier breakdown [15, 16]. An accumulation of leukocytes in glomeruli has also been reported in human DN and animal DN models [17], suggesting a potential role of inflammatory responses in the glomerular capillary destruction. One of the angiopoietin molecules, angiopoietin 1, has been reported as a potent anti-inflammatory factor, inhibiting plasma penetration and ICAM-1-mediated inflammatory infiltration in the development of diabetic retinopathy [18, 19]. In our experiments, the occurrence of leukocyte infiltration in the capillary endothelial cells was obviously lower in those patients with a pronounced elevation in ANGPTL2 mRNA. Immunohistochemical study also indicated a negative relationship between the upregulated ANGPTL2 protein and the presence of monocyte infiltration. Thus, we propose that this factor may possess an analogous anti-inflammatory effect, as does angiopoietin 1, which is very important in diabetic microvascular injury.

The present study is the first to investigate the expression characteristics of ANGPTL2 in human DN. Our studies are based on functional genomic screening and expressional analysis in human samples. On the basis of these observations, we propose that ANGPTL2 is a novel angiogenesis-associated regulator and anti-inflammatory modulator in diabetic glomerulopathy. The future determination of its functions may lead to a better understanding of the complex pathogenesis of DN.

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