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Quantification of glomerular TGF-β1 mRNA in patients with diabetes mellitus

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Quantification of glomerular TGF-β1 mRNA in patients with diabetes mellitus. Transforming growth factor-β1 (TGF-β1) is a primary determinant of the mesangial expansion observed in diabetic nephropathy. In this study, we quantitated the levels of intraglomerular TGF-β1 mRNA in patients with diabetes mellitus using a competitive polymerase chain reaction (PCR) method. Renal biopsy specimens were obtained from 29 patients with non-insulin-dependent diabetes mellitus. Total RNA was extracted from the glomeruli and reverse transcribed into cDNA with reverse transcriptase. To prepare samples containing identical amounts of β-actin cDNA (8 pg), we performed competitive PCR by co-amplifying mutant templates of β-actin with a unique *Eco*RI site. We also used this competitive PCR method to measure TGF-β1 cDNA by co-amplifying mutant templates of TGF-β1. We observed higher expression of TGF-β1 mRNA in glomeruli of patients with diabetic nephropathy as compared with normal glomeruli. Intraglomerular TGF-β1 mRNA was elevated, even in the early stage of diabetic nephropathy. Moreover, levels of intraglomerular TGF-β1 mRNA correlated with values of HbA1c. These data suggest that hyperglycemia induces intraglomerular TGF-β1 mRNA expression *in vivo*, and that TGF-β1 overproduction may be associated with the progression of diabetic nephropathy.

Diabetic nephropathy is a fatal complication of diabetes mellitus and a common cause of end-stage renal disease [1]. It is characterized histologically by expansion of the glomerular mesangium due to an increase in extracellular matrix (ECM) proteins [2]. Although hyperglycemia plays a key role in the initiation and progression of diabetic nephropathy, the mechanisms by which hyperglycemia causes the histologic change remain to be elucidated.

There have been several reports indicating that various cytokines and vasoactive peptides were associated with the pathogenesis of diabetic nephropathy. Atrial natriuretic peptide increased glomerular filtration rate [3]. Overproduction of platelet-derived growth factor B (PDGF-B), tumor necrosis factor-α (TNF-α), and basic fibroblast growth factor (bFGF), all of which are mitogenic for cultured mesangial cells, has been observed in diabetic rats [4]. Transforming growth factor-β1 (TGF-β1) may be a primary determinant of mesangial expansion observed in diabetic nephropathy for the following reasons. Firstly, Yamamoto et al have demonstrated enhanced expression of TGF-β1 in the glomeruli of diabetic rats and localization of the TGF-β1 protein in patients with diabetic nephropathy by immunohistochemistry [5]. Secondly, Wolf et al have demonstrated that hyperglycemia stimulats TGF-β1 synthesis in cultured mesangial cells [6]. Thirdly, Choi et al have shown that rat mesangial hypertrophy is induced when exposed to TGF-β1 *in vitro* [7].

Quantitative analysis of the glomerular mRNA encoding TGF-β1 in cases of human diabetic nephropathy is difficult since few renal biopsy specimens are available. Peten et al have shown that human glomerular mRNA encoding for α2 (IV) collagen can be measured by a competitive polymerase chain reaction (PCR) method [8]. Our previous study have also demonstrated that levels of glomerular TGF-β1 mRNA can be measured accurately by a competitive PCR method [9]. In this study, we quantitated glomerular expression of TGF-β1 mRNA in patients with diabetic nephropathy to determine whether TGF-β1 plays a role in the progression of diabetic nephropathy. Furthermore, we investigated the correlation between the levels of intraglomerular TGF-β1 mRNA and several clinical parameters.

Methods

Patients

Renal biopsy specimens were obtained from 29 patients with non-insulin-dependent diabetes mellitus (NIDDM). All patients gave their informed consent. Table 1 summarizes the clinical characters of these patients.

We obtained control specimens from 11 patients (8 males and 3 females) with renal cell cancer. The mean age of the patients with renal cell cancer was 58 ± 10 years (mean ± SD). The mean values of creatinine clearance were 91 ± 22 ml/min (mean ± SD). The control specimens, which consisted of the remaining normal kidney after resections for renal cell cancer, showed only minor changes in glomeruli by light microscopy (normal glomeruli).

Light microscopy

Renal biopsy specimens for light microscopy were fixed in 10% neutral phosphate-buffered formalin, embedded in paraffin and cut into 3 μm sections. The sections were stained with the periodic acid-Schiff reagent. Patients were divided into three groups (Diffuse I to Diffuse III) based on the extent of mesangial expansion

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Table 1. Characteristics of patients

Patient number	Age/sex	Duration *years*	Complication	Urinary albumin	Histology	C_{Cr} *ml/min*	TGF-β1 *fg//pg β-actin*
1	64/M	3		normo	Diffuse I	78	0.88
2	62/M	3	hypertension hyperlipidemia	normo	Diffuse I	96	0.25
3	60/F	3		normo	Diffuse I	97	1.01
4	60/M	3		normo	Diffuse I	76	0.54
5	62/M	3	hypertension	normo	Diffuse I	79	0.22
6	58/M	4		micro	Diffuse I	93	2.00
7	46/F	4	hypertension	micor	Diffuse II	125	1.50
8	51/F	6	hypertension hyperlipidemia	normo	Diffuse II	133	1.50
9	46/F	6		normo	Diffuse I	80	0.40
10	45/M	6		micro	Diffuse I	86	1.00
11	69/M	7		overt	Diffuse II	45	0.70
12	62/M	8		normo	Diffuse I	83	0.40
13	55/F	9		overt	Diffuse I	118	0.45
14	57/F	9	hyperlipidemia	micro	Diffuse III	82	1.00
15	61/F	10	hypertension	normo	Diffuse II	56	0.50
16	62/M	10	hypertension	overt	Diffuse III	89	0.25
17	64/F	11		normo	Diffuse III	50	0.50
18	61/M	13		normo	Diffuse I	89	1.27
19	65/M	15	hypertension	normo	Diffuse II	48	0.25
20	44/F	15		overt	Diffuse III	36	1.25
21	68/M	16		overt	Diffuse III	25	2.00
22	69/F	16	hypertension	normo	Diffuse II	67	0.50
23	50/M	18		overt	Diffuse III	40	0.75
24	61/M	20		overt	Diffuse III	48	1.53
25	69/M	20		overt	Diffuse III	39	0.32
26	43/F	20	hyperlipidemia	micro	Diffuse III	63	0.25
27	45/F	21		micro	Diffuse I	56	1.00
28	67/F	25		micro	Diffuse II	143	3.00
29	67/M	30		normo	Diffuse I	90	2.00

Abbreviations are: M, male; F, female; duration, duration of disease; normo, normoalbuminuria; micro, microalbuminuria; overt, overt albuminuria; Diffuse I-III are defined in **Method's** section; C_{Cr}, creatinine clearance.

according to the Gellman's classification (Fig. 1) [10], with the exception that the Diffuse III group in this study contained both the Diffuse III and Diffuse IV groups defined by the Gellman's classification.

Immunohistochemical localization of type IV collagen

Paraffin sections (4 μm) were deparaffinized and endogenous peroxidase activity was denatured with 1% hydrogen peroxide. After incubation for five minutes at 37°C in 0.4 mg/ml proteinase K (Dako Japan Co., Kyoto, Japan), the sections were irradiated with microwaves for two minutes. Sections were subsequently incubated with 10% normal goat serum, then reacted with a polyclonal antibody to human type IV collagen (Chemicon International Inc., Temeula, CA, USA) for two hours at 37°C. After washing with phosphate-buffered saline (PBS), the sections processed further using LSAB kits (Dako). The staining intensity of collagen type IV was graded semiquantitatively as weak (Grade I), moderate (Grade II), and strong (Grade III).

Immunohistochemical localization of TGF-β1

Frozen sections (3 μm) were fixed in cold aceton for 10 minutes before staining. After washing with PBS, sections were reacted with a polyclonal antibody to human TGF-β1 (King Brewing Co., Kakogawa, Japan) for two hours at 37°C. After washing with PBS, the sections were incubated with fluorescein-conjugated F(ab')2 goat anti-rabbit IgG (Cappel, Malvern, PA, USA), which had been absorbed with normal human plasma before use. For nuclear staining, propydium iodide (Sigma, St. Louis, MO, USA) was used. The section thus prepared was examined with a laser confocal microscope (MRC-600, Bio-Rad, Tokyo, Japan).

Total RNA extraction and reverse transcription

Glomeruli were obtained by dissecting 10 to 20% of each of the renal biopsy specimens under phase-contrast microscopy as described previously [11]. We extracted total RNA from glomeruli as described previously [9]. Total RNA was reverse transcribed into cDNA with 1 unit/μl of RAV-2 reverse transcriptase (Amersham, Aylesbury, UK). The reaction mixture also contained 100 pM of random hexamers, 1 mM of dATP, dCTP, dTTP, and dGTP, and 1 U/μl RNase inhibitor (Amersham). The reaction mixtures was incubated at 42°C for one hour, then heated to 95°C for five minutes to denature the RNA-cDNA hybrid and to inactivate the reverse transcriptase.

Oligonucleotide primers

The primers were synthesized on a PCR-Mate DNA synthesizer (Applied Biosystems, Inc., Foster, CA, USA). Human β-actin cDNA was amplified with the following primers: TGACGGGGTCACCCACACTGTGCCCATCTA (bases 509 to 538) as the sense primer and ACTCGTCATACTCCTGCTTGCTGATCCA (bases 1107-1134) as the antisense primer [12]. Human TGF-β1

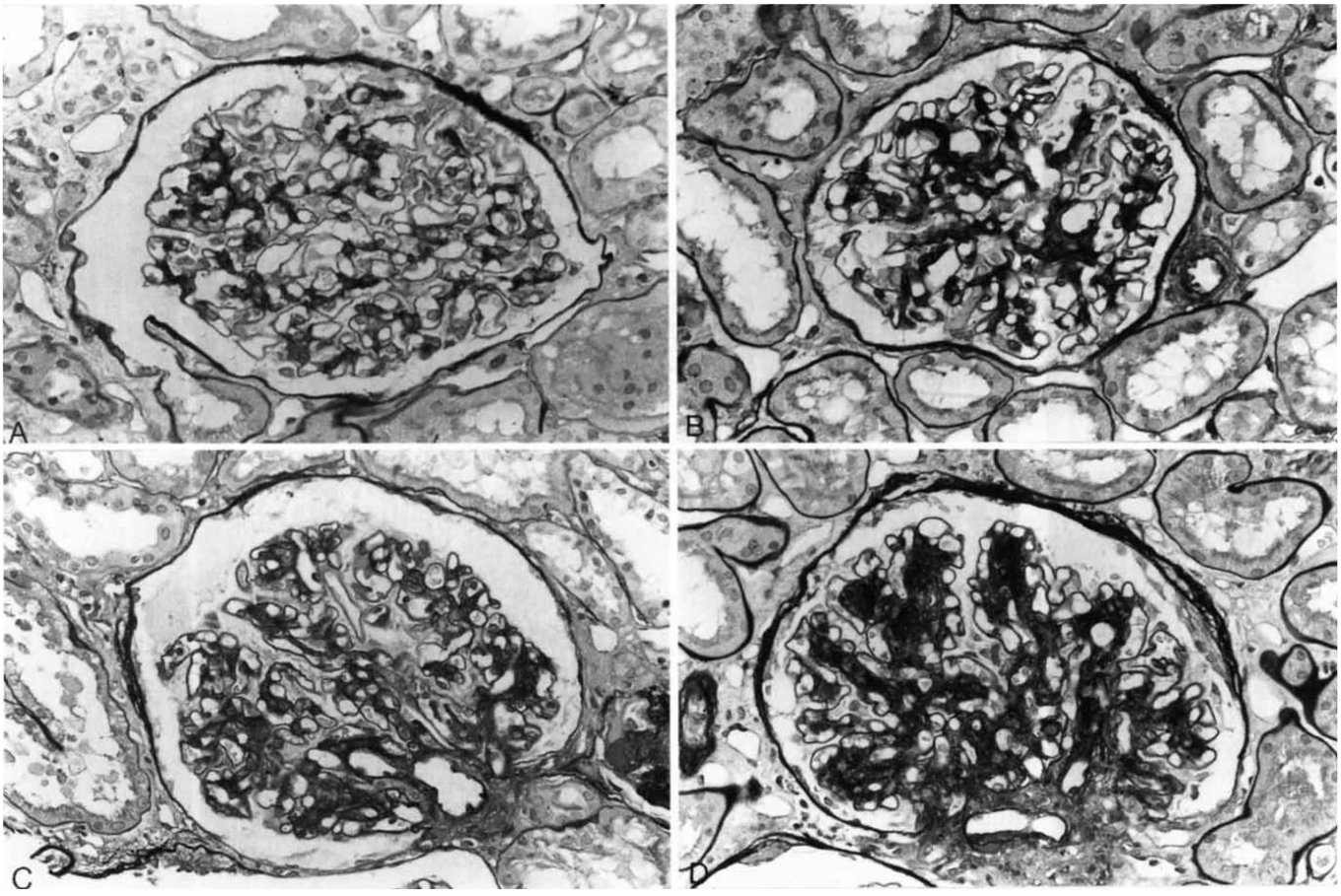

Fig. 1. *A glomerulus showing segmental widening of the mesangial area (Diffise I) (upper left, periodic acid-Schiff staining, × 400).* Mild enlargement of the mesangial area is observed in a diffuse pattern (Diffuse II) (upper right, periodic acid-Schiff staining, × 400). The mesangial area is increased and the capillary wall is diffusely thickened. Some capillary lumina are reduced in size due to the mesangial enlargement (Diffuse III) (bottom left, periodic acid-Schiff staining, × 400). Mesangial expansion is marked. In Diffuse IV, nodules are occasionally observed (bottom right, periodic acid-Schiff staining, × 400).

cDNA was amplified with the following primers: AGCGACTC-GCCAGAGTGGTTATCTT (bases 1411-1435) as the sense primer and TTATGCTGGTTGTACAGGGCCAGGA (bases 1860-1884) as the antisense primer [13]. These primers were derived from separate exons to inhibit amplification of any contaminating genomic DNA sequences.

Generation of competitive mutant templates

To circumvent the problem of variability in amplification efficiency, competitive templates were generated by site specific mutagenesis using two mutant primers as described by Higuchi, Krummel and Saiki [14]. Mutant primers 30 base pairs in length contained a single base pair change which created a unique *Eco*RI site in the competitive templates of the human β-actin gene and the human TGF-β1 gene. Mutant fragments were subcloned into pUC 18 and verified by sequencing [9].

Competitive PCR

Competitive PCR was performed as described by Gilliland, Perrin and Bunn [15]. A master mix containing primers, PCR buffer, *Taq* polymerase (Perkin Elmer/Cetus, Norwalk, CT, USA) and an appropriate amount of cDNA was prepared. Ten microliters of the competitive template at various concentrations were added to 90 μl of the above mixture. The mixture was overlaid with mineral oil and then amplified with the Perkin-Elmer/Cetus thermal cycler. The amplification profile was the following: denaturation at 95°C for one minute, primer annealing at 65°C for 30

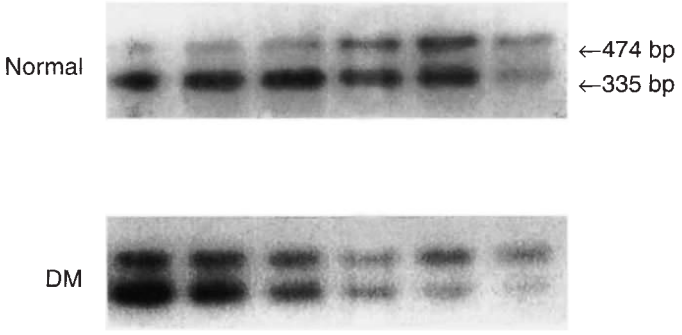

Fig. 2. *Southern blot of amplified sequences by competitive PCR.* Upper bands (474 bp) were amplified from the authentic TGF-β1. Lower bands (335 bp) were amplified from the mutant TGF-β1 template. The starting concentrations of competitive mutant template in each tube (left to right) were 8, 6, 4, 2, 1, 0.5 fg in normal glomeruli (Normal) and 36, 30, 24, 18, 12, 6 fg in glomeruli of diabetic patients (DM), respectively.

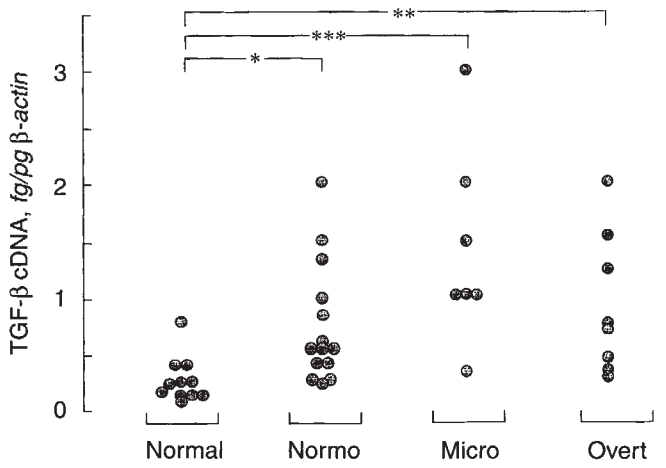

Fig. 3. *Levels of intraglomerular TGF-β1 mRNA and urinary albumin excretion.* $*P < 0.01$, $**P < 0.005$, $***P < 0.001$. Patients were divided into three groups according to their excretion of urinary albumin: patients with normoalbuminuria (normo), patients with microalbuminuria (micro), and patients with overt albuminuria (overt).

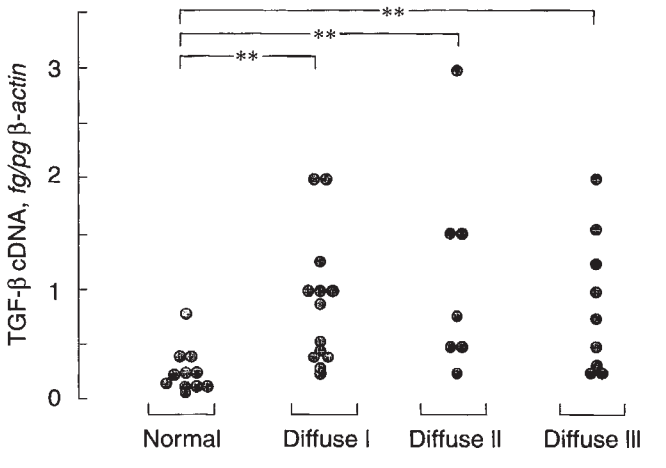

Fig. 4. *Levels of intraglomerular TGF-β1 mRNA and the extent of mesangial expansion in renal biopsy.* $**P < 0.005$. Patients were divided into three groups based on the extent of mesangial expansion seen on renal biopsy.

seconds and extension at 72°C for one minute. After 40 amplification cycles, an aliquot of each sample was digested with *Eco*RI and run on a 2% NuSieve/agarose gel (FMC Bioproducts, Rockland, ME, USA). To exclude cDNA contamination, total RNA was directly amplified with 1 pg of β-actin mutant template or 1 fg of TGF-β1 mutant template. No authentic bands were observed when the total RNA was directly amplified.

The amount of glomerular β-actin cDNA was determined by densitometric analysis of ethidium bromide staining intensity.

Southern blot hybridization

Southern blot analysis was used to determine the amount of glomerular TGF-β1 cDNA. DNA underwent electrophoresis, denaturation and transfer to a Hybond-N+ filter (Amersham). After drying at 80°C for two hours, the filters were prehybridized in 6 × SSC, 10 × Denhardt's solution, 1% SDS and 50 μg/ml salmon sperm DNA at 42°C for three hours. They were then hybridized with a oligonucleotide probe (GGTAGTGAACCCG-TTGATGTCCACTTGCA) that had been 5′ end labeled with [γ-^{32}P] ATP. This probe was complementary to sequence of exon 4 and 5 of the TGF-β1 gene. The filters were hybridized at 37°C overnight, washed with 6 × SSC and 0.1% SDS at room temperature three times for 10 minutes and at 60°C for 30 minutes. After exposure to Kodak X-Omat film (Eastman Kodak Co., Rochester, NY, USA), the amount of cDNA was analyzed by densitometry.

We performed competitive PCR on 10 samples on five different days and found interassay variation that was consistently below 15%.

Statistical analysis

To compare means among more than two groups, but the underlying distribution was not assumed to hold normal, a multiple comparison test for a nonparametric, the Kruskal-Wallis test was used. When the average ranks of the groups were judged unequal by the test, the Dunn procedure was followed for a comparison of specific groups. All data were expressed as the median (25th percentile, 75th percentile). Correlations were assessed by linear regression analysis. P value < 0.05 was considered to be statistically significant.

Results

Quantitative analysis of intraglomerular TGF-β1 in patients with diabetes mellitus

The initial competitive PCR allowed us to determine the amount of intraglomerular β-actin cDNA. We found that the levels of intraglomerular β-actin cDNA were significantly higher in the patients with diabetic nephropathy [13.4 (7.7, 16) pg/glomerulus] than in the normal glomeruli [7.2 (4.2, 11.5) pg/glomerulus; $P < 0.001$]. Therefore, we should adjust the samples to equal concentrations of β-actin cDNA. We used samples containing 8 pg of β-actin cDNA to determine the amount of intraglomerular TGF-β1 mRNA. We again performed competitive PCR by co-amplifying the mutant templates of TGF-β1. Figure 2 shows the representative result of competitive PCR in normal glomeruli and glomeruli obtained from patients with diabetes mellitus. Raising amounts of the mutant TGF-β1 cDNA increasingly inhibited amplification of authentic TGF-β1 cDNA. The ratio of mutant to authentic band density was calculated.

Levels of intraglomerular TGF-β1 mRNA were significantly elevated in patients with diabetes mellitus as compared with levels in normal glomeruli [0.75 (0.4, 1.33) fg/pg β-actin vs. 0.2 (0.13, 0.36) fg/pg β-actin, $P < 0.001$]. Patients were divided into three groups according to their excretion of urinary albumin: 13 patients with normoalbuminuria, 7 patients with microalbuminuria (> 15 μg/min), and 9 patients with overt albuminuria (> 200 μg/min) [16]. The amounts of intraglomerular TGF-β1 mRNA in patients with normoalbuminuria, microalbuminuria and overt albuminuria were 0.50 (0.40, 1.00), 1.00 (1.00, 1.88), 0.73 (0.39, 1.39) fg/pg β-actin, respectively. TGF-β1 mRNA levels were significantly elevated in patients from all groups compared to normal glomeruli (Fig. 3). Patients were also divided into three groups based on the extent of mesangial expansion seen on renal biopsy (Diffuse I to Diffuse III). The amounts of intraglomerular TGF-β1 mRNA

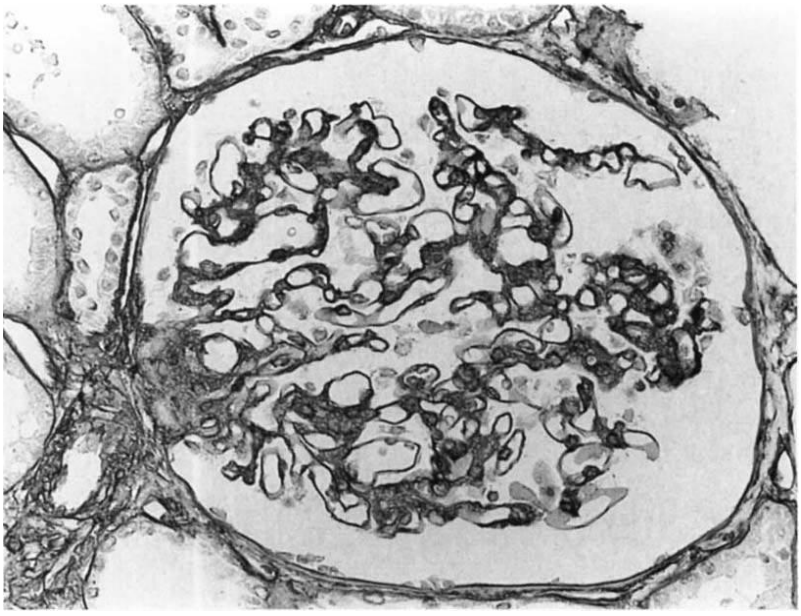

Fig. 5. *Immunohistochemical localization of collagen type IV.* Positive immunostaining for collagen type IV was observed in the mesangium, glomerular basement membrane, and Bowman's capsule (× 400).

in the Diffuse I, the Diffuse II and the Diffuse III group were 0.88 (0.40, 1.07), 0.7 (0.5, 1.5), and 0.75 (0.30, 1.32) fg/pg β-actin, respectively. TGF-β1 mRNA levels were significantly elevated in patients from all groups compared to normal glomeruli (Fig. 4). These results indicate that high levels of glomerular TGF-β1 mRNA can be observed even in the early stage of diabetic nephropathy.

Correlation between glomerular TGF-β1 mRNA and staining intensity of collagen type IV

To examine the correlationship between the levels of intraglomerular TGF-β1 mRNA and collagen type IV synthesis, we performed immunostaining with anti-collagen type IV in 27 patients with diabetic nephropathy. Positive immunostaining for collagen type IV was observed in the mesangium, glomerular basement membrane, and Bowman's capsule (Fig. 5). The levels of intraglomerular TGF-β1 mRNA were positively correlated with the staining intensity of collagen type IV (Fig. 6).

Immunohistochemical localization of TGF-β1 in diabetic nephropathy

To elucidate the production of TGF-β1 protein in glomeruli, we performed immunostaining with anti-TGF-β1. Frozen sections were obtained from 7 patients with diabetes mellitus (patient number 3, 4, 7, 17, 21, 24, 28 in Table 1). Control specimens were obtained from five patients with renal cell cancer. Positive staining were observed in the mesangial area as shown in Figure 7. All of the glomeruli from the seven patients with diabetes mellitus were strongly positive for TGF-β1, whereas glomeruli from the control specimens showed only trace staining. The staining were negative when the primary antibody was preabsorbed with purified TGF-β1 or when the tissues were incubated with the secondary antibody alone.

Correlation between glomerular TGF-β1 mRNA and clinical parameters

Next, we investigated correlation between intraglomerular TGF-β1 mRNA and several clinical parameters. Values for

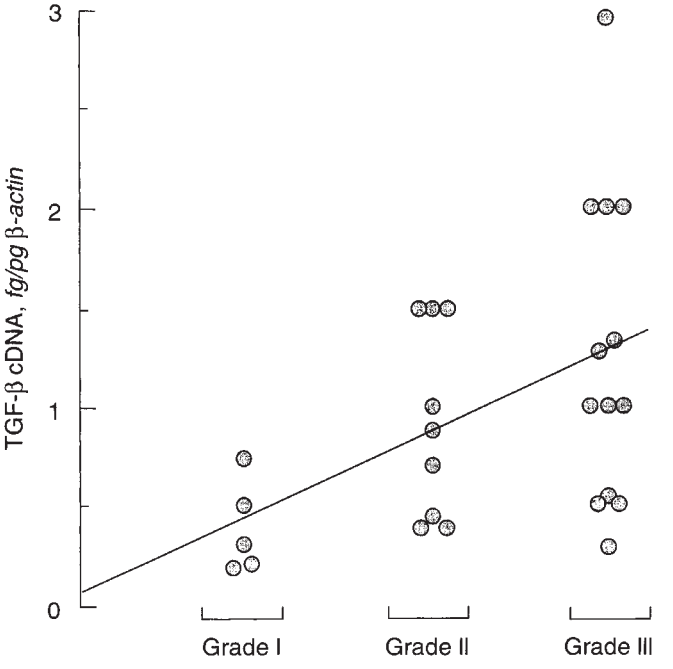

Fig. 6. *Correlation between levels of intraglomerular TGF-β1 mRNA and the staining intensity of collagen type IV.* The levels of intraglomerular TGF-β1 mRNA were positively correlated with the staining intensity of collagen type IV.

intraglomerular TGF-β1 mRNA correlated well with HbA1c values ($r = 0.65$, $P < 0.001$; Fig. 8). However, values for glomerular TGF-β1 mRNA levels did not correlate with values for other clinical parameters such as urinary albumin excretion, urinary β2 microglobulin excretion, urinary NAG excretion nor endogenous serum creatinine. In addition, no correlation was found with the following parameters: presense of hypertension or hyperlipidemia, and duration of disease.

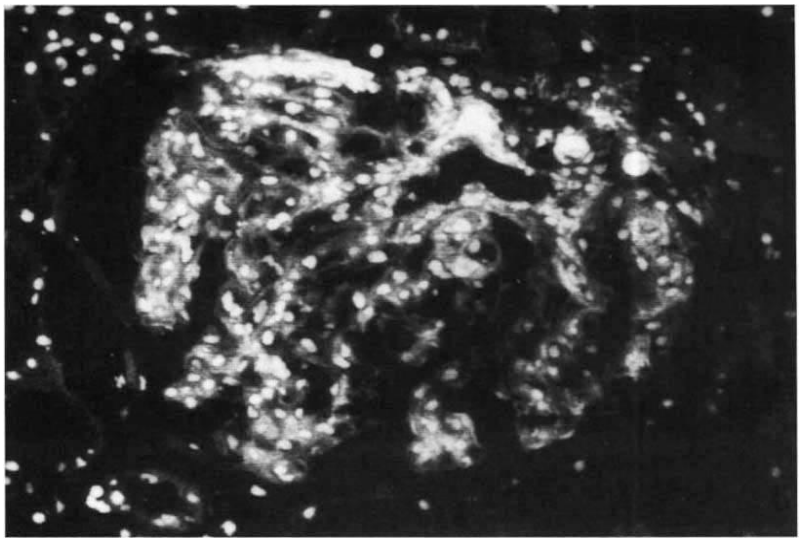

Fig. 7. *Confocal laser micrograph of a glomerulus from diabetes stained with anti-TGF-β1.* Positive staining was observed in the mesangium (× 400).

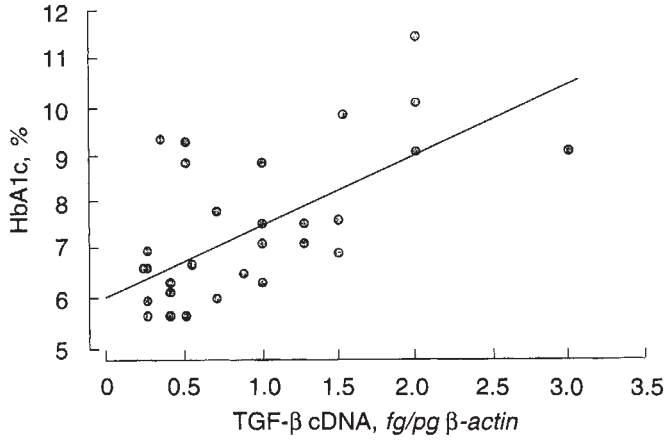

Fig. 8. *Correlation between levels of intraglomerular TGF-β1 mRNA and HbA1c.* Values for intraglomerular TGF-β1 mRNA correlated well with HbA1c values.

Discussion

Quantifying the expression of mRNA can provide important information about protein levels and thus biological activity. We used competitive PCR to measure the expression of intraglomerular TGF-β1 mRNA in patients with diabetic nephropathy. This procedure uses a competitive mutant template of the same sequence as the target except for the addition of a restriction site by a single base substitution. Accordingly, differences in amplification efficiency should be negligible. However, there are two other significant problems in quantitating the intraglomerular expression of mRNA; a difficulty in quantitating the amount of total RNA and an inconsistency in the efficiency of reverse transcription. To circumvent these probrems, we first quantified the amounts of mRNA expression of the "housekeeping" gene, β-actin, and then diluted the samples so they contained equal amounts of β-actin cDNA.

Levels of glomerular TGF-β1 mRNA can be measured accurately by a competitive PCR method. However, we cannot identify which cells in glomeruli are responsible for TGF-β1 production by a competitive PCR method. Previous studies have shown that TGF-β1 protein and mRNA are mainly observed in mesangial area [5, 17, 18]. Therefore, TGF-β1 is thought to be mainly produced by mesangial cells in glomeruli.

TGF-β1 functions in tissue repair by stimulating the synthesis of extracellular matrix and also inhibiting its degradation [19, 20]. However, overproduction of TGF-β1 can lead to fibrosis via the pathologic accumulation of extracellular matrix [17]. Isaka et al have shown that introduction of the TGF-β1 gene into glomerular cells induces mesangial matrix expansion *in vivo* [21]. Border et al have demonstrated that inhibition of TGF-β1 activity prevents the accumulation of the mesangial matrix in antithymocyte serum-induced glomerulonephritis [22, 23]. Several studies have shown that TGF-β1 is closely associated with mesangial matrix increase in diabetic rat, mouse and human glomeruli [5–7, 24–31]. These previous reports suggest that TGF-β1 may be a main determinant of mesangial expansion observed in diabetic nephropathy. However, the prior data in cell culture, animal experiments and human tissue analysis have all focused on insulin-dependent diabetes mellitus (IDDM). We have speculated the same association between TGF-β1 and diabetic nephropathy due to NIDDM. We found that significantly higher levels of intraglomerular TGF-β1 mRNA were observed in patients with NIDDM and intraglomerular TGF-β1 mRNA levels were elevated even in the early stage of diabetic nephropathy. In addition, TGF-β1 protein was localized in the glomeruli of patients with NIDDM. Accordingly, it is conceivable that overproduction of TGF-β1 in glomeruli is associated with the initiation and progression of diabetic nephropathy due to NIDDM as well as IDDM.

TGF-β1 is secreted as a biologically latent form. Therefore, TGF-β1 mRNA levels may not parallel TGF-β1 activity. In this study, intraglomerular levels of TGF-β1 mRNA were correlated with the amounts of glomerular collagen type IV. As mature TGF-β1, which is functionally active, induces collagen type IV synthesis in cultured mesangial cells [26, 31], this result provides the indirect evidence that latent TGF-β1 is activated in the glomeruli of the patients with diabetic nephropathy.

Shankland and Scholey have demonstrated that TGF-β1 expression increases in glomeruli in the early phase of renal

hypertrophy and normalization of blood glucose levels with insulin treatment attenuates the increase in TGF-β1 expression in diabetic rats [32]. Strict glycemic control has been shown to ameliorate the progression of human diabetic nephropathy [33]. The present study indicated that values for intraglomerular TGF-β1 mRNA correlated well with HbA1c values. These data suggest that glycemic control slow the progression of diabetic nephropathy partly via the inhibition of TGF-β1 production in glomeruli. To clarify the association between glycemic control and the inhibition of TGF-β1 production, we should perform the follow-up study and measure the intraglomerular TGF-β1 mRNA after strict glycemic control.

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