# Activation of Transforming Growth Factor-$\beta_1$ in Diabetic Kidney Disease

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**Recent data have suggested that certain growth factors and cytokines are involved in the development of diabetic nephropathy. The aim of this study was to investigate whether circulating transforming growth factor beta 1 (TGF-$\beta_1$) and tumor necrosis factor alpha (TNF-$\alpha$) are associated with diabetic kidney disease. Serum levels of active and total TGF-$\beta_1$ and TNF-$\alpha$ were measured in type 2 diabetic patients with nephropathy (n = 23) or without (n = 35) and normoglycemic controls (n = 12). Serum levels of circulating active TGF-$\beta_1$ were significantly higher in patients with diabetic nephropathy (0.43 ± 0.06 ng · mL$^{-1}$) compared with diabetic patients without renal involvement (0.23 ± 0.03 ng · mL$^{-1}$, $P = .002$) and healthy controls (0.24 ± 0.03 ng · mL$^{-1}$, $P = .001$), whereas the levels of total (active + latent) TGF-$\beta_1$ were not different between the subgroups. Active TGF-$\beta_1$ concentrations were correlated with urinary albumin excretion ($r = .49$, $P < .003$) and serum creatinine ($r = .55$, $P < .01$). Sera from patients with type 2 diabetes contained significantly more TNF-$\alpha$ than sera from normoglycemic controls (3.07 ± 0.24 *v* 1.65 ± 0.20 pg · mL$^{-1}$, $P = .001$). However, the comparison of serum TNF-$\alpha$ concentrations between microalbuminuric and normoalbuminuric diabetic patients showed no significant difference (3.21 ± 0.28 *v* 2.97 ± 0.34 pg · mL$^{-1}$, $P = .12$). In conclusion, type 2 diabetic patients with diabetic nephropathy exhibit increased activation of TGF-$\beta_1$ in serum, suggesting an association between circulating TGF-$\beta_1$ activity and the development of renal disease.**


DATA FROM THE UK Prospective Diabetes Study have demonstrated that hyperglycemia is associated with the development of diabetic nephropathy in patients with diabetes mellitus type 2, suggesting that the metabolic consequences of hyperglycemia may induce structural changes in the diabetic kidney.[1,2] The pathomorphologic changes during renal involvement in diabetes include thickening of the glomerular and tubular basement membranes, hypertrophy of both tubuloepithelial and glomerular structures, and deposition of extracellular matrix components in the glomerular mesangium.[2-4] However, the basic process mediating the effect of chronic hyperglycemia on the development of diabetic nephropathy is incompletely understood.

Recent reports suggest that transforming growth factor beta (TGF-β) plays a critical role in the pathogenesis of diabetic kidney disease.[5-18] TGF-β, a multifunctional cytokine, is secreted by virtually all cell types in a latent biologically inactive form consisting of dimers of both the precursor remainder, designated as the latency-associated peptide (LAP), and mature TGF-$\beta_1$ and is activated proteolytically by serine proteases in vivo.[19,20] Among its many actions, activated TGF-β influences cell growth and matrix production in an autocrine and/or paracrine fashion.[21] TGF-β is involved in the regulation of glomerular endothelial, epithelial, and mesangial proliferation and induces hypertrophy of mesangial cells.[16,22-24] In addition, TGF-β is known to promote extracellular matrix accumulation by glomerular epithelial cells, glomerular mesangial cells, and tubular epithelial cells.[25-27] Mesangial and proximal tubular cells cultured in media containing high glucose concentrations show increased production of TGF-$\beta_1$ mRNA and protein followed by enhanced collagen biosynthesis.[12,14] Increased TGF-$\beta_1$ mRNA, protein, and bioactivity in the kidney have also been demonstrated in several animal models of diabetes.[7,9-11,15,16] Furthermore, increased expression of TGF-$\beta_1$ has been demonstrated in renal biopsies from patients with established diabetic nephropathy.[11,18,28] Kopp et al reported that a 6- to 8-fold elevation of circulating active TGF-$\beta_1$ in a transgenic mouse model was associated with progressive glomerular disease characterized by mesangial expansion, accumulation of glomerular immune deposits and extracellular matrix proteins, and interstitial fibrosis.[29] Since the histomorphological changes observed in that study are comparable to those that are characteristic of diabetic kidney disease,[3-5] we hypothesized that increased activation of circulating TGF-$\beta_1$ may be associated with the development of diabetic nephropathy.

Recent data have strongly suggested that tumor necrosis factor alpha (TNF-α) plays a central role in mediating insulin resistance in non–insulin-dependent diabetes mellitus (NIDDM). TNF-α, a pluripotent inflammatory cytokine, is produced by a variety of cell types, including neutrophils, activated lymphocytes, macrophages, and fat cells. TNF-α is implicated in initiating the cascade of other cytokines and factors that comprise the immune system's response to infection and sometimes cancer.[30,31] An analysis of several animal models of obesity and NIDDM showed that TNF-α is best correlated with massive obesity and insulin resistance. Enhanced expression of TNF-α mRNA and elevated TNF-α protein levels, locally as well as systematically, were reported in these studies.[29,32]

Recent investigations have demonstrated that TNF-α also can contribute to the pathogenesis of renal disease. In lupus nephritis, significant expression of TNF-αmRNA has been observed.[29] In animal models of diabetic nephropathy, glomerular TNF-α expression correlated with the progression of diabetic nephropathy.[15,33] Thus, a causal association of TNF-α activity with diabetic kidney disease has been proposed.[15,33] In patients with type 1 diabetes mellitus, as well as type 2, elevated levels of circulating TNF-α have been reported.[34,35] However, larger studies comparing TNF-α concentrations of diabetic patients with or without renal disease have not been performed. To assess whether circulating levels of TNF-α and TGF-$\beta_1$ are

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altered in diabetic kidney disease, we measured the serum concentrations of these cytokines in type 2 diabetic patients with or without diabetic nephropathy and healthy controls.

## SUBJECTS AND METHODS

### *Patients*

Fifty-eight consecutive hospitalized type 2 diabetic patients who agreed to participate and 12 nondiabetic control subjects were studied. All subjects provided informed consent. The study protocol was approved by the ethics committee of the medical faculty of Ruhr University, Bochum, Germany.

Type 2 diabetic patients with and without diabetic nephropathy were analyzed separately. These two subgroups were matched for sex, age, and weight. Patients and controls with severe chronic or inflammatory diseases, rheumatoid disease, liver disease, or severe illness were excluded. The diabetic patients did not have peripheral ulcers. Diabetic nephropathy was defined by albuminuria repeatedly greater than 30 mg/d, proteinuria greater than 250 mg/d, or serum creatinine greater than 132 μmol/L. The medical history, physical examination, urinalysis, and abdominal sonography showed no evidence for renal diseases other than diabetic nephropathy as a possible cause of proteinuria or impaired renal function in any of the subjects. Retinopathy (defined as background retinopathy) and neuropathy were diagnosed based on clinical examination by specialty physicians. Peripheral vascular and cerebrovascular disease was determined by clinical history (intermittent claudication/stroke) and Doppler echography. Coronary heart disease (CHD) was determined by electrocardiogram (ECG), stress ECG, history of myocardial infarction, or coronary angiography.

Blood was sampled from 8 to 9 AM into cooled serum tubes and immediately placed on ice. The serum was allowed to clot and then centrifuged within 30 minutes at 4°C at 2,500× *g* for 15 minutes.

### *Determination of TGF-$\beta_1$*

TGF-$\beta_1$ was determined with a quantitative sandwich-enzyme immunoassay specific for TGF-$\beta_1$ using the extracellular domain of the TGF-$\beta_1$ receptor obtained from R&D Systems (Minneapolis, MN). Each serum sample was assayed in duplicate for active and total (latent + active) TGF-$\beta_1$. Since the antibody (TGF-$\beta_1$ receptor) only recognizes the active form of TGF-$\beta_1$, the latent form is detected only by prior acidification of the sample to separate the LAP from the bioactive TGF-$\beta_1$ dimer. Activation was achieved by adding 1 mol · $L^{-1}$ HCl for 15 minutes at room temperature followed by neutralization with 1.2 mol · $L^{-1}$ NaOH while controls of pH were performed. This procedure reduces the pH in the samples to approximately 2.6 and then returns it to approximately 7.6 upon neutralization. The assay does not cross-react with TGF-$\beta_2$ or TGF-$\beta_3$, interleukins 1 to 11, fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF) A-chain and B-chain heterodimer, interferon gamma, or TNF-α and -β. The intraassay and interassay coefficient of variation at 100 pg · $mL^{-1}$ was 4% and 7%, respectively. The range was between 16 and 1,000 pg · $mL^{-1}$. Recombinant TGF-$\beta_1$ was used as a standard. The assay was calibrated using a standard from the National Institute for Biological Standards and Control ([NIBSC] South Mimms, UK).

### *Determination of TNF-α*

Total serum TNF-α, both free and bound to soluble TNF-α receptors (TNF-α-Rs), was determined with a high-sensitivity quantitative sandwich-enzyme immunoassay obtained from R & D Systems. The assay does not cross-react with interleukins 1 to 11, granulocyte colony-stimulating factor (CSF), granulocyte-macrophage CSF, TGF-$\beta_1$, basic and acidic FGF, PDGF-AB, interferon-gamma, erythropoietin, TNF-β, TNF-α-RI, or TNF-α-RII and is not affected by the presence of soluble TNF-α-RI or -RII. The intraassay and interassay coefficient of variation at 8 pg/mL was 5.8% and 8.6%, respectively. The concentration range of the assay was 0.4 to 20 pg · $mL^{-1}$, and all values were within this range. Recombinant *Escherichia coli*–expressed TNF-α was used as a standard, which yields parallel standard curves to the First International Standard NIBSC. One picogram per milliliter is equivalent to 0.86 NIBSC (87/650) units.

### *Statistical Analysis*

Data are expressed as the mean ± SEM. The Kolmogorov-Smirnov *Z* test was used to analyze the distribution of the samples. Values for TGF-β, TNF-α, and high- and low-density lipoprotein cholesterol were not normally distributed. The Kruskall-Wallis test was used to compare non–parametrically distributed values between more than two groups, and the Mann-Whitney test to compare values between two groups. In the case of a normal distribution, statistical significance was tested using Student's *t* test and ANOVA in the case of more than two groups. Regression analysis was used to calculate Spearman correlation coefficients. Statistical analysis was performed using SPSS for Windows version 7.5 (SPSS, Chicago, IL).

## RESULTS

The study population included 58 type 2 diabetic patients and 12 healthy controls. Patients with diabetic nephropathy had a longer duration of diabetes and higher levels of hemoglobin $A_{1c}$ ($HbA_{1c}$) and serum creatinine. The number of patients treated with insulin, an oral antidiabetic, or both was comparable in both subgroups (Table 1).

There was no difference in the level of active TGF-$\beta_1$ in sera from type 2 diabetic patients and controls (0.29 ± 0.03 and 0.24 ± 0.03 ng · $mL^{-1}$, respectively, $P = .7$). Serum levels of total (active + latent) TGF-$\beta_1$ tended to be higher in patients with type 2 diabetes (10.8 ± 2.1 and 9.5 ± 2.1 ng · $mL^{-1}$, respectively), but this was not statistically different ($P = .35$) compared with the nondiabetic population (Fig 1). However, the mean value for total TGF-$\beta_1$ is derived from a very asymmetric data distribution (data not shown) and is reflected in the large standard deviation. Diabetic patients with and without diabetic nephropathy exhibited similar amounts of circulating total TGF-$\beta_1$ ($P = .69$; Fig 1A). In contrast, serum levels of circulating active TGF-$\beta_1$ were significantly higher in patients with diabetic kidney disease (0.43 ± 0.06 ng · $mL^{-1}$) compared with diabetic patients without renal involvement (0.23 ± 0.03 ng · $mL^{-1}$, $P = .002$). Figure 1B illustrates that in addition, serum levels of active TGF-$\beta_1$ were correlated with urinary albumin excretion ($R^2 = .49$, $P < .003$). This correlation was independent of age, sex, and diabetes duration. Furthermore, the amount of TGF-$\beta_1$ in the biologically active form was correlated with serum creatinine levels ($R^2 = .55$, $P < .01$). There was no correlation for active or total TGF-$\beta_1$ with $HbA_{1c}$, fasting glucose, and cardiovascular risk markers including cholesterol, triglycerides, and body mass index, or with any medication like insulin, oral antidiabetic agents, aspirin, and antihypertensive agents including angiotensin-converting enzyme (ACE) inhibitors (data not shown). The occurrence of diabetic neuropathy, retinopathy, macrovascular disease, and CHD was not associated with increased serum levels of active

**Table 1. Clinical Characteristics of the Study Population**

| Characteristic | Healthy Controls (n = 12) | NIDDM Patients | | |
|---|---|---|---|---|
| | | Total (n = 58) | Nephropathy (n = 23) | No Nephropathy (n = 35) |
| Age (yr) | 65.4 ± 1.1 | 66.3 ± 2.6 | 67.7 ± 1.4 | 64.0 ± 1.6 |
| No. of males | 5 (42%) | 24 (41%) | 8 (34%) | 16 (45%) |
| Diabetes duration (yr) | — | 9.5 ± 0.9 | 12.8 ± 1.7 | 7.3 ± 1.4§ |
| Insulin therapy (%) | — | 31 | 34 | 28 |
| Oral antidiabetics (%) | — | 67 | 69 | 65 |
| ACE inhibitors (n) | 4/12 (48%) | 35/58 (60%) | 16/23 (69%) | 19/35 (54%) |
| Waist to hip ratio | 1.10 ± 0.1 | 1.10 ± 0.2 | 1.09 ± 0.08 | 1.11 ± 0.09 |
| Body mass index ($kg/m^2$) | 27.3 ± 0.6 | 28.4 ± 2.0 | 28.0 ± 1.0 | 26.8 ± 0.8 |
| Creatinine (μmol/L) | 87.5 ± 10.8 | 91.5 ± 19.3 | 99.8 ± 5.3 | 86.0 ± 1.9‖ |
| Albuminuria (mg/d) | 13.8 ± 4.7 | 243.3 ± 96* | 596.6 ± 226 | 11.1 ± 1.1‖ |
| Thrombocyte count (per nL) | 259.8 ± 22 | 234.8 ± 8.2 | 234.4 ± 4.3 | 235.2 ± 1.1 |
| Fasting blood glucose (mmol/L) | 4.5 ± 0.2 | 8.15 ± 0.5† | 9.47 ± 0.9 | 7.85 ± 0.5 |
| $HbA_{1c}$ (%) | 5.16 ± 0.1 | 7.43 ± 0.2‡ | 8.08 ± 0.3 | 7.00 ± 0.2‖ |

NOTE. Data are the mean ± SEM.
*$P < .05$, †$P < .01$, ‡$P < .001$, NIDDM (total) *v* controls.
§$P < .02$, ‖$P < .01$, nephropathy *v* no nephropathy.

or total TGF-$\beta_1$. There was no difference between TGF-$\beta_1$ serum levels (active and total) in male and female subjects in each subgroup (data not shown).

Figure 2 shows that serum levels of TNF-α were significantly higher in diabetic patients ($3.07 \pm 0.24$ pg · $mL^{-1}$) compared with controls ($1.65 \pm 0.2$ pg · $ml^{-1}$, $P = .001$). Furthermore, circulating TNF-α correlated with $HbA_{1c}$ ($R^2 = .49$, $P < .001$) and serum fructosamine ($R^2 = .29$, $P < .02$). Sera from patients with present or abundant diabetic nephropathy contained similar amounts of TNF-α ($3.21 \pm 0.28$ *v* $2.97 \pm 0.34$ pg · $mL^{-1}$).

There was no association for serum TNF-α levels with urinary albumin excretion, diabetic kidney disease or other complications, and cardiovascular risk factors (data not shown). TNF-α serum levels of male and female subjects were not statistically different in diabetic patients ($3.28 \pm 0.48$ *v* $2.92 \pm 0.28$ pg · $mL^{-1}$) and normoglycemic controls ($1.53 \pm 0.24$ *v* $1.74 \pm 0.31$ pg · $mL^{-1}$) in each subgroup.

## DISCUSSION

The results of the present study show that the activation of circulating TGF-$\beta_1$ is markedly increased in type 2 diabetic patients with diabetic nephropathy compared with diabetics without renal involvement and healthy controls. The elevation of active TGF-$\beta_1$ in patients with diabetic kidney disease correlated significantly with urinary albumin excretion and serum creatinine levels.

This is the first study to show increased serum levels of active TGF-$\beta_1$ in human diabetic nephropathy. In two recent studies, circulating active TGF-$\beta_1$ was almost unmeasurable and was not different between NIDDM patients and controls.[5,36] However, the finding of very low levels of active TGF-$\beta_1$ in these studies may be due to underlying CHD, since all patients either were scheduled for elective cardiac catheterization (Sharma et al[5]) or had a higher prevalence of already established CHD (Pfeiffer et al[36]) compared with the present investigation (49% *v* 28%, respectively). In fact, Grainger et al[28,37] found depressed serum levels of active TGF-$\beta_1$ in patients with CHD, while sera from healthy subjects and patients without CHD contained significant amounts of circulating active TGF-$\beta_1$. Furthermore, in both studies, the percentage of type 2 diabetic patients with

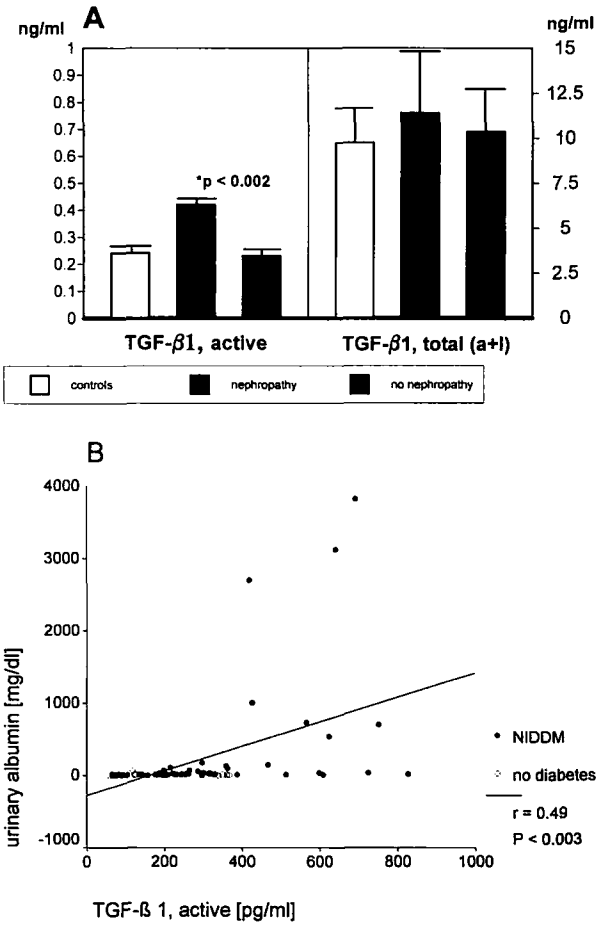


**Fig 1. (A) Serum concentrations of active and total (active + latent) TGF-$\beta_1$ in type 2 diabetic patients with (n = 23) and without (n = 35) diabetic nephropathy and normoglycemic controls (n = 12). *Significant difference for type 2 diabetic patients with nephropathy *v* type 2 diabetic controls without nephropathy and controls. Data are the mean ± SEM. (B) Correlation of circulating active TGF-$\beta_1$ with urinary albumin in NIDDM patients and normoglycemic probands. Solid line indicates linear regression line between circulating active TGF-$\beta_1$ and urinary albumin.**

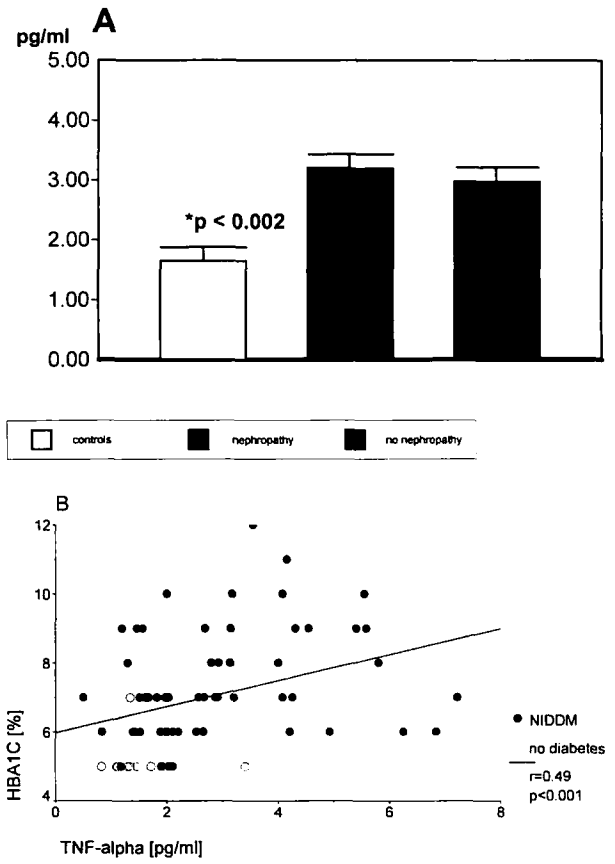


**Fig 2. (A) Serum concentrations of TNF-α in patients with type 2 diabetes with (n = 23) and without (n = 35) diabetic nephropathy and normoglycemic controls (n = 12). *Significant difference for type 2 diabetic patients (with and without renal disease) *v* normoglycemic controls. Data are the mean ± SEM. (B) Correlation of serum TNF-α with $HbA_{1c}$ in NIDDM patients and normoglycemic controls. Solid line indicates linear regression line between circulating TNF-α and $HbA_{1c}$.**

renal involvement was lower, since a comparison of microalbuminuric and nonmicroalbuminuric patients was not the primary study endpoint.[5,36] Finally, the use of serum samples instead of plasma in our study may not explain the divergent results, as concentrations of total (latent + active) and biologically active TGF-β have been proposed to be very similar in serum and platelet-poor plasma prepared from the same blood sample.[37] Even in the case of contamination caused by the release of TGF-$\beta_1$ from platelets, the levels of latent TGF-$\beta_1$ are more likely to be affected than the process of activation. In addition, possible contamination would affect all groups simultaneously and would not explain the different amounts of activated TGF-$\beta_1$. In agreement with a previous study,[36] the levels of total (active + latent) TGF-$\beta_1$ tended to be higher in patients with type 2 diabetes than in the nondiabetic population, but this difference did not reach statistical significance. However, the mean value for total TGF-$\beta_1$ is derived from a very asymmetric data distribution (data not shown) and is reflected in the large standard deviation. Thus, in view of the small sample size, statistical significance is likely to be missed. Treatment of rat mesangial cells in culture with angiotensin II was shown to increase the production of TGF-β.[38] In addition, reduced levels of TGF-β glomerular protein and mRNA and collagen type I and III after treatment with an ACE inhibitor or angiotensin II receptor antagonist were found in a rat model of glomerulosclerosis.[39] It may be hypothesized from these reports that the use of ACE inhibitors might affect circulating TGF-$\beta_1$ levels, especially in renal disease. However, our results did not show any significant correlation between the use of ACE inhibitors and serum levels of active or total TGF-$\beta_1$. In fact, the effect of modulating the angiotensin system in diabetic renal disease might be different versus other models of glomerulosclerosis, as glucose, but not angiotensin II, was shown to stimulate the production of TGF-β and fibronectin by glomerular epithelial cells.[40]

The factors influencing the activation of TGF-$\beta_1$ in vivo are incompletely understood. Thus, a discussion on the possible causes for increased TGF-$\beta_1$ activity in diabetic nephropathy has to be largely speculative. Circulating TGF-$\beta_1$ is secreted as a biologically inactive, non–covalently bound complex consisting of dimers of both the precursor remainder (LAP) and mature TGF-$\beta_1$. While mature TGF-$\beta_1$ can be released from the complex in active form in vitro by a variety of treatments including acidification and proteolytic cleavage, the exact mechanism for activation of latent TGF-$\beta_1$ in vivo is not known.[38] Cell-derived plasminogen activators (PAs) convert plasminogen to plasmin, which in turn activates latent TGF-β proteolytically at or near the cell surface, allowing its binding to the receptor.[41] It was demonstrated that the binding of TGF-β to the cell surface leads to the production of plasminogen activator inhibitor (PAI) in vitro.[42] PAI inhibits the further activation of plasminogen, resulting in a negative feedback of plasmin-mediated TGF-β activation.[41] Since TGF-$\beta_1$ itself is a potent inducer of PAI-1 production, increased activation of circulating TGF-$\beta_1$ in diabetic nephropathy should be followed by enhanced PAI-1 activity. In fact, a study by Gruden et al[43] found increased PAI-1 activity in microalbuminuric diabetic patients compared with normoalbuminuric patients. The high PAI-1 activity and the fact that circulating PA, a strong promoter of TGF-$\beta_1$ activation, was not higher in microalbuminuric compared with normoalbuminuric diabetic patients[43] suggest that the higher levels of circulating activated TGF-$\beta_1$ in diabetic nephropathy result from local rather than systemic activation. Cell-associated components of the plasmin-antiplasmin system are thought to be involved in local activation of TGF-$\beta_1$.[44] Therefore, the degree of local TGF-β activation depends on the balance of locally expressed activating and inactivating factors and thus is tissue-specific. It is unknown at present which cells and tissues promote increased local activation of TGF-β. Results from a study in murine mesangial cells suggest that glucose may be a direct activator of latent TGF-β.[45] Mesangial and proximal tubular cells cultured in media containing high glucose concentrations exhibit increased production of TGF-$\beta_1$ mRNA and protein followed by enhanced collagen biosynthesis, suggesting an increased bioactivity of TGF-$\beta_1$.[11,13] Alternatively, glucose may indirectly increase active TGF-β levels via the release of proteases like plasmin. Metabolic changes due to impairment of renal function might affect the activation of TGF-β, since serum creatinine and active TGF-$\beta_1$ correlated

significantly in this study. However, none of the factors presently known to be responsible for TGF-β activation have been shown to be influenced by mild renal dysfunction. Finally, increased serum levels of active TGF-$\beta_1$ are unlikely to result from a reduction of glomerular filtration, since active TGF-$\beta_1$, a small molecule with a molecular weight of only 25 kd, is expected to cross the glomerular capillary blood-urine barrier.[5,20] Net renal extraction of total TGF-β has been reported in nondiabetic humans.[5]

In agreement with the findings of the present study, elevated concentrations of circulating TGF-β have been demonstrated in patients with other fibrotic disorders such as hepatic and pulmonary fibrosis, suggesting a causative role of TGF-β in the pathogenesis of fibrotic disorders such as progressive diabetic nephropathy.[46] Since renal biopsies were not studied in the present investigation, our results provide indirect, but not direct, evidence for a causal association of increased serum levels of active TGF-$\beta_1$ and fibrosis of renal tissue in patients with diabetes mellitus type 2. However, such a direct correlation linking increased TGF-β activity in the circulation with the development of diabetic nephropathy has been demonstrated in several animal models of diabetes.[47-49] Kopp et al[49] reported that a 6- to 8-fold elevation of circulating active TGF-$\beta_1$ in a transgenic mouse model was associated with progressive glomerular disease characterized by mesangial expansion, accumulation of glomerular immune deposits and extracellular matrix proteins, and interstitial fibrosis. In another study, increased serum activity of TGF-$\beta_1$ was associated with enhanced glomerular collagen synthesis in streptozotocin (STZ)-induced diabetic rats.[48] In STZ-induced diabetic mice, neutralization of TGF-β by anti–TGF-β antibodies attenuated renal hypertrophy and enhanced extracellular matrix gene expression, suggesting a causal association of TGF-β activity and kidney disease in diabetes.[50] Since the histomorphological changes observed in these studies are comparable to those that are characteristic of the diabetic kidney,[3,4] increased activation of circulating TGF-$\beta_1$ may contribute to the development of diabetic nephropathy. Increased expression of TGF-$\beta_1$ has been demonstrated in renal biopsies from patients with established diabetic nephropathy[10,17,27] and in several animal models of diabetes.[6,8-10,14,15] The biological activity of TGF-β in the glomerular microenvironment responsible for progressive fibrosis depends on the relative abundance of mature TGF-β.[16] Besides local production of TGF-$\beta_1$ in the kidney, due to its low molecular weight of 25 kd, circulating activated TGF-$\beta_1$ is expected to be able to enter the kidney from the circulation and thus promote the development of diabetic renal disease, whereas the larger molecular complex of latent protein-bound TGF-β is unlikely to cross the glomerular filtration barrier.[49] If additional factors like increased synthesis of glomerular angiotensin II, formation of advanced glycation end products, and elevated glomerular capillary pressure are present, initial glomerular disease is likely to progress. Since several of these contributing factors have been shown to enhance the activity of TGF-β itself,[51,52] it has been assumed that the cumulative effect may be a pronounced increase in TGF-β activity leading to excessive matrix deposition and clinical diabetic nephropathy.[5]

Based on several experiments in animal models of diabetes, it has been suggested that TNF-α may play a role in the development of diabetic nephropathy.[15,33] In two recent studies, elevated levels of circulating TNF-α in patients with type 2 diabetes have been reported.[34,35] However, both studies did not address the role of TNF-α in diabetic renal disease specifically. In agreement with previous studies,[34,35] we found increased concentrations of circulating TNF-α in patients with NIDDM compared with normoglycemic controls. In this study, serum concentrations of TNF-α were significantly correlated with $HbA_{1c}$. Thus, the results from the present investigation in conjunction with evidence from previous studies in animals and type 2 diabetic patients suggest a correlation of circulating TNF-α and glycemic control in NIDDM.[29,32,34,35] However, in the present investigation, the comparison of serum TNF-α levels in patients with and without diabetic nephropathy showed no difference, suggesting that a causative role of circulating TNF-α in the development of diabetic kidney disease is unlikely.

In fact, several lines of evidence argue against an endocrine role of TNF-α in NIDDM. First, although elevated plasma levels of TNF-α have been measured in obese *db/db* mice, as well as NIDDM patients,[34,35,53] the absolute levels of 20 to 200 pg/mL observed in mice and 3 to 6 $ng \cdot mL^{-1}$ in humans (present study and Hotamisligil and Spiegelmann[53]) are well below those required to suppress insulin action in cultured cell systems.[32] This is not surprising, since the level of TNF-α required to activate TNF-α receptors is much higher than that found in the circulation.[31] Second, total serum and plasma levels of TNF-α measured by ELISA may even overestimate the amount of circulating TNF-α that is biologically active.[54] In fact, soluble TNF-α receptors (p55 and p75) have been shown to correlate with circulating TNF-α and to inhibit its biological activity.[54] These data suggest that TNF-α present in serum is not relevant as a circulating cytokine, but must be regarded as an overflow from cell types producing this cytokine. Thus, circulating levels of TNF-α may provide an index of local cytokine activity.

The source of local production responsible for the increased serum TNF-α in diabetes is unclear. Expression of TNF-α mRNA in adipose tissue was suggested to be associated with hyperinsulinemia and the body mass index (BMI).[53] However, in agreement with a previous study,[34] circulating TNF-α and the BMI were unrelated in the present investigation, suggesting that tissues apart from fat may contribute to serum TNF-α. In animal models of diabetes, as well as type 2 diabetic patients, stimulated macrophages were shown to release more TNF-α versus their normoglycemic counterparts.[55] Thus, release of TNF-α by macrophages may be responsible, at least in part, for elevated serum TNF-α levels in diabetes.

In summary, the results of the present study demonstrate that diabetic nephropathy in type 2 diabetic patients is associated with elevated serum levels of activated TGF-$\beta_1$. Further long-term longitudinal studies are required to determine whether the degree of activation of circulating TGF-$\beta_1$ can be used as a marker for patients at risk for diabetic nephropathy and/or its progression. The TGF-β system may be a new therapeutic target for the treatment and prevention of diabetic nephropathy.

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