Accurate Measurement and Clinical Significance of Urinary Transforming Growth Factor-Beta₁

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Key Words

Transforming growth factor- β_1 • Enzyme immunoassay • Proteinuria • Glomerular injury

Abstract

Transforming growth factor β_1 (TGF- β_1) is the main modulator of the healing process after tissue injury. In the kidney, if TGF- β_1 release is not switched off, extracellular matrix components (ECM) are accumulated and tissue fibrosis occurs. Urinary TGF- β_1 levels reflect its renal production and it has been determined in various types of glomerular disease. In this review, a critical analysis of the different immunoassays that have been used for the measurement of TGF- β_1 in the urine is presented and the importance of the serial determination of urinary TGF- β_1 levels in patients with various types of renal disease is discussed.

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Introduction

Transforming growth factor- β (TGF- β) represents a group of 25-kDa proteins, which are actively involved in the development and differentiation of various tissues [1]. TGF- β was originally isolated from platelets, but it has also been found in cell cultures of monocytes/macrophages and mesangial cells [2]. Three isoforms of TGF- β

have been identified in mammalian species: TGF- β_1 , TGF- β_2 and TGF- β_3 . TGF- β_1 , the most important isoform in humans, is secreted from cells in the form of a high-molecular-weight latent complex [3]. Cleavage of this larger precursor molecule is necessary for TGF- β_1 activation [2, 3]. TGF- β_1 targets specific receptors in various cell types and often exerts its biological effects through autocrine and paracrine pathways [2, 3].

TGF- β_1 is the main modulator of the healing process after tissue injury [4]. Normally, its release ceases by feedback mechanisms, when the healing process has been completed [3, 4]. However, if TGF- β_1 release is not switched off, extracellular matrix components (ECM) are accumulated and tissue fibrosis occurs [4]. Increased TGF- β_1 synthesis in the glomeruli and tubulointerstitial area has been identified in rats with subtotal nephrectomy and other types of experimentally induced renal disease [5, 6]. Upregulation of TGF- β_1 synthesis in the kidney is followed by accumulation of collagen and scarring, whereas the administration of specific antiserum against TGF- β_1 results in amelioration of renal damage [7]. Increased synthesis and decreased degradation of ECM components, activation of interstitial fibroblasts and differentiation of tubular epithelial cells into myofibroblasts as well as apoptosis of tubular epithelial cells are stimulated by TGF- β_1 and contribute to the development of renal scarring and to the loss of intrinsic renal cells [8–10]. Although its exact role in the progression of human renal

disease has not been established, accumulating evidence suggests that TGF- β_1 is actively involved, since increased synthesis of TGF- β_1 m-RNA has been described in the renal tissue of patients with various types of chronic nephropathies [11, 12].

Plasma TGF- β_1 levels, measured in patients with various types of renal disease, were found to be similar with those of healthy subjects. Elevated plasma TGF- β_1 levels decreased with the administration of angiotensin II receptor antagonists (ARA) and have been described in renal allograft recipients [13, 14]. However, serum levels of the growth factor may reflect not only renal produced, but also platelet-derived TGF- β_1 after platelet degranulation. Thus, estimation of platelet activation in the blood samples is necessary [15].

Urinary TGF- β_1 levels, measured in patients with glomerulonephritis and diabetic nephropathy, as well as in renal allograft recipients, were found to be higher than in healthy subjects [16–19]. Increased amounts of TGF- β_1 in the urine have been observed, along with increased excretion of other proteins, such as retinol-binding protein and α_1 -microglobulin that are considered indicators of tubular epithelial cells injury or dysfunction [19, 20]. These findings, along with the detection of TGF- β_1 mRNA and protein within the tubular epithelial cells in patients with various types of renal disease, suggest that urine TGF- β_1 excretion reflects its intrarenal production [21]. In this review, a critical analysis of the methods measuring TGF- β_1 is presented and the importance of its determination in the urine, in relation to the evolution of the disease and to response to treatment is discussed, in patients with different types of renal disease.

Determination of TGF- β_1 in Urine

Methods for Measuring TGF- β_1

Urinary TGF- β_1 levels were initially determined by commercially available kits based on enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody [22, 23]. Later on, some laboratories developed their own assays [17, 24]. However, conflicting results have been reported, in patients with various types of renal diseases and this was due to different assays (kits, antibodies and standards), as well as to the various methods used for the activation of TGF- β_1 in the urine. Since TGF- β monomers associate with a latent protein [25], the complex needs to be activated by an acidification – alkalization process, in order to determine TGF- β .

Today, several kits for the measurement of TGF- β_1 are commercially available and its determination is based upon sandwich immunoassays, by using either precoated plates with TGF- β_1 soluble receptor or anti-human TGF- β_1 (as it can be obtained from the internet, e.g. www.bio-compare.com). A polyclonal anti-human TGF- β_1 , either biotinylated or HRP conjugated, is used as the detection antibody. It is of note that most of the commercially available kits have been designed to detect TGF- β_1 in serum samples or culture fluids, according to their instruction. However, some of these kits have been used for measurement of urinary TGF- β_1 , after modifications that have been introduced by some laboratories in the activation process of TGF- β_1 [20, 26]. In Europe, the cost of these kits ranges between EUR 450 and 650 (USD 550–750).

Building an EIA for the Measurement of TGF- β_1 Concentration

In our laboratory, we have focused our interest on the renal expression of TGF- β_1 and its urinary excretion [12, 21]. We initially tried a commercially available kit for determination of the excreted TGF- β_1 . Although our results on its plasma concentration were in agreement with other studies, we noticed a disagreement between urinary TGF- β_1 levels and renal expression of this growth factor. The kit was quite expensive and since the results were questionable, we decided to develop an easily reproducible double antibody enzyme immunoassay according to Honkanen et al. [17]. This immunoassay was based on the formation of a sandwich complex of TGF- β_1 with a specific mouse monoclonal and a rabbit polyclonal antibody. The immune complex, in a double-antibody sandwich immunoassay, gives a stronger signal than the competitive ELISA, which uses only one antibody. Thus, lower concentrations of the antigen can be determined and the assay becomes more sensitive. Modifications, depended on the abilities and the supplies of our laboratory, were also made. The building up procedure for the final EIA which is been used in our laboratory [21, 27, 28], is described below.

Flat-bottomed, high-binding microtiter plates (96 wells) were used (Costar, USA, Cat. No. 2581). Alternatively, plates with 12 strips (8 wells each) and the same properties may also be used (Costar, USA, Cat. No. 3590). Wells were coated with 100 μ l of mouse monoclonal anti-TGF- β antibodies, 2 μ g/ml (Genzyme Diagnostics, USA, code: 80-1835-03) in 0.05 M Na₂CO₃ buffer pH 9.0, by overnight incubation, at 4°C. The wells were filled and washed with PBS, 0.05% Tween-20, four times. Consequently, 200 μ l Super Block blocking buffer (Pierce, USA)

were added to all wells – to avoid nonspecific interactions – and incubated for 3 h at room temperature. Super Block was removed, wells were washed and dried and could then be stored at 4°C until assay. On the day of the assay, 100 µl of undiluted samples were acid-activated with 50 µl 1 N HCl and incubated for 2 h at 4°C. Samples were neutralized with the addition of approximately 50 µl 1.2 N NaOH/0.5 M HEPES and pH was checked by adding 2 µl of the neutralized sample on a pH strip. 100 µl of standard dilutions (R&D Systems, UK) and 100 µl of neutralized samples were incubated in the wells overnight at 4°C. The TGF-β₁ bound onto the wells was then detected with a rabbit polyclonal anti-TGF-β₁ antibody (R&D Systems, UK) labeled with horseradish peroxidase (200 µl, 1.5 h at RT). Peroxidase activity was determined using tetramethylbenzidine (TMB), as a substrate (R&D Systems, UK). To calculate intra-assay precision, three samples were tested 5 times each on one plate. To calculate interassay precision same samples were tested in 5 separate assays. The intra- and interassay coefficients of variation (CV) were 7.4 and 6.3%, respectively. The recovery of TGF-β₁ standards (50 and 100 pg/ml) ranged from 83 to 112% and the detection limit was 5 pg/ml. To calculate recovery of TGF-β, three aliquots of the same urine sample were used and 5 and 2.5 µl from the 2,000 pg/ml standard was added, respectively, in the two of them, thus increasing their concentration by 100 and 50 pg/ml. TGF-β was determined in the three samples and the recovery was calculated by assuming that their difference should be 100 and 50 pg/ml, respectively. The procedure was performed in triplicate. To determine detection limit of the assay, concentrations less than 20 pg/ml were used by further diluting standards at concentrations of 10 and 5 pg/ml. Urinary TGF-β₁ excretion was expressed in ng/24 h and plasma TGF-β₁ concentration in pg/ml. Urine samples were not spiked with albumin to repeat the recovery experiments. It was taken for granted that proteinuria does not interfere in the assay, since the secondary antibody and the standards that we used, are also used for the determination of TGF-β in the plasma, where the protein concentration is much higher than in the urine.

Alternatively, an unlabeled rabbit polyclonal anti-TGF-β₁ antibody (sc-146) can be used (1:1,000), together with the ABC staining system with similar variation coefficients, recovery and detection limit.

Buying or Building an EIA System?

A question arises, for a laboratory that wants to determine urinary TGF-β₁ excretion, whether buying a commercially available kit (money consuming – time saving)

or using a home made EIA (time consuming – money saving). Since both types of EIAs are reliable, the answer will be based on the balance between cost and time consumption of each laboratory.

As stated above, the cost of an EIA, 96 tests, ranges between EUR 450 and 650. At the same cost the monoclonal antibody for coating the microtiter plates and the polyclonal antibody for the detection of the immunocomplex can be purchased in quantities enough for 4 × 96 tests. Although the development of an EIA system is time consuming, it might be money saving in research laboratories where microtiter plates, blocking solutions, PBS, ABC staining system, and substrate solution are parts of its routine. However, the purchase of these secondary but necessary reagents for just one EIA system might be both money and time consuming for a typical clinical laboratory in a General Hospital. In addition, experienced personnel are required in order to build the assay and evaluate its variation coefficients and results.

In table 1, comparable data are presented from data sheets of some commercially available kits, modified or not, as well as from homemade EIAs.

Urinary TGF-β₁ Excretion in ng/mg Creatinine or ng/24 h?

The unit, which the urinary TGF-β₁ excretion should be determined with, is another issue that needs to be discussed. The ratio of TGF-β₁ over creatinine in urine (pg/mg creatinine), in random or 24-hour urine collections, used to represent the most commonly used way for the quantization of TGF-β₁ urinary excretion. Others have measured TGF-β₁ in a 3-hour morning urine collection and expressed its excretion in pg/ml. We believe that the determination of TGF-β₁ levels in 24-hour urine collection and its expression in ng/24 h, rather than of pg/mg creatinine, are more accurate. This opinion is based on the fact that TGF-β₁ is not related with the urinary creatinine excretion, which differs among patients with normal renal function and those with renal insufficiency. Besides, if we compared two groups of patients, with the same urinary TGF-β₁ excretion, in ng/24 h, but with different creatinine clearance and creatinine excretion, the calculation and use of the ratio pg TGF-β₁/mg creatine will be misleading, as it will provide varying results. In addition, by expressing urinary TGF-β in ng/24 h we do not allow any possible daily variations of TGF levels to interfere in our results. Due to the above, we are convinced that the best way to express urinary TGF-β₁ is in ng/24 h, as is the case with proteinuria (g/24 h).

Table 1. Comparison of variation coefficients among several commercially available kits and homemade EIAs

	Intra-assay coefficient, %	Interassay coefficient, %	Recovery %	Lower detection limit, pg/ml
Antigenix America	7.3	11.7	not found	9
BD Biosciences	not found	not found	not found	4
R&D Systems	7.5	12.2	81–123	7
Promega	3.1	11.6	97	15
Ellis et al. [20]*	8.5	13.1	92–96	not reported
Agarwal et al. [26]*	2.7	4.8	94	0.7
Honkanen et al. [17]	5.9	8.1	87–103	5
Goumenos et al. [21]	7.4	6.3	83–112	5

* Modified R&D Systems assay kit.

Urinary TGF- β_1 Levels in Patients with Various Types of Renal Disease

IgA Nephropathy

Increased urinary TGF- β_1 excretion has been reported in patients with IgA nephropathy [16, 17, 29–33]. A significant correlation of these levels with the degree of mesangial proliferation, matrix expansion and crescent formation, has also been described [16, 30, 32]. A significant reduction of urinary TGF- β_1 levels was observed with the administration of corticosteroids and angiotensin II receptor blockers (ARBs) [16, 29, 31–33]. This effect of corticosteroids and ARBs is possibly related to TGF- β secretion during the inflammatory process in the kidney and to its activation by proteinuria and angiotensin II per se.

Angiotensin II acts as a local growth factor, stimulating cellular hypertrophy and collagen type IV synthesis, in proximal tubular cells, whereas its administration in cultured mesangial cells, increases the levels of active TGF- β_1 [32, 34]. Urinary TGF- β_1 was significantly reduced in patients with IgA nephropathy, proteinuria (>1 g/24 h) and impaired renal function after a few weeks of treatment with losartan, but not with amlodipine, although both treatments regulated blood pressure in a similar degree [29, 31]. Although the reduced with treatment urinary TGF- β_1 levels were higher than those observed in healthy subjects, ARBs may provide a beneficial effect by blocking the production of renal TGF- β_1 .

Crescentic Nephritis

Elevated urinary TGF- β_1 levels have been observed in patients with crescentic nephritis [16, 27, 32]. Extremely high levels were observed in patients who showed no im-

provement of renal function with immunosuppressive therapy (steroids and cyclophosphamide) [27]. Patients who improved their renal function with treatment had significantly lower, but still higher levels than those observed in healthy subjects. Urinary TGF- β_1 excretion remained high even after administration of immunosuppressive drugs, in all patients with improved or stabilized renal function [27].

Elevated urinary TGF- β_1 activity has been observed, in experimentally induced anti-GBM (glomerular basement membrane) nephritis, very early in the course of the disease. The time course was identical to that observed for the renal cortical production of TGF- β_1 and it was related to the development of fibrotic lesions [35]. Activation and proliferation of various cells types, via cytokines and growth factors, are involved in the development of crescents [36–41]. The administration of immunosuppressive therapy during the development of cellular crescents can restrict glomerular injury, whereas the development of fibrous crescents suggests a permanent damage. TGF- β_1 is an immune regulator and its increased urinary excretion, during the early course of crescentic nephritis, may represent an index of acute glomerular inflammation. The observation of extremely high urinary TGF- β_1 levels in patients with deteriorating renal function may underline the development of irreversible renal damage in these patients.

Membranous Nephropathy

Increased urinary TGF- β_1 levels have been reported in nephrotic patients with membranous nephropathy [17, 21, 42]. A significant correlation of these levels with urinary albumin excretion rate and severity of histological involvement (interstitial inflammation and sclerosis in-

dex) has been observed [17]. Patients with persistent nephrotic syndrome and/or declining renal function had significantly higher TGF- β_1 levels in comparison to patients entering remission, whereas a decrease of urinary TGF- β_1 was noted after administration of steroids or cytotoxic drugs along with a reduction of proteinuria and stabilization of serum creatinine [17].

Elevated TGF- β_1 levels in the urine of nephrotic patients with membranous nephropathy decreased upon remission of nephrotic syndrome with steroids and cyclosporine [21]. TGF- β_1 mRNA and protein expression were also studied in biopsy sections of these patients, using *in situ* hybridization and immunohistochemistry. TGF- β_1 was localized within tubular epithelial cells, renal interstitium and to a lesser extent within the glomeruli of nephrotic patients, whereas no TGF- β_1 was detected in control biopsies. The extent of TGF- β_1 immunostaining was related to the degree of interstitial inflammation and fibrosis [21].

These findings suggest an upregulation of renal TGF- β_1 by filtered protein. It is well known that heavy proteinuria represents an independent risk factor for poor outcome in most types of glomerular disease. The uptake of filtered protein from tubular epithelial cells is followed by activation of genes coding for vasoactive and chemoattractant peptides that contribute to the accumulation of monocytes and myofibroblasts in the renal interstitium leading to inflammation and fibrosis [43–45].

Diabetic Nephropathy

Increased urinary TGF- β_1 excretion has been reported in patients with insulin-dependent diabetes mellitus (IDDM) and non-IDDM [20, 32, 46–51]. A significant correlation of these levels with urinary excretion of collagen type III (T3C) and retinol-binding protein (RBP) (markers of extracellular matrix synthesis and tubular injury or dysfunction) has been reported in IDDM patients [20]. Urinary TGF- β_1 levels were reported higher in patients with diabetic nephropathy, compared to those of normoalbuminuric IDDM patients with the same duration of diabetes and levels of HbA1c [47, 48]. A decrease of these levels along with lower HbA1c levels was observed after a better metabolic control [52, 53].

Increased urinary concentration of the TGF- β inducible gene h3 (Big-h3), a protein used to assess the biological activity of TGF- β in various tissues, has been described in a large number of non-IDDM patients and correlated with stage of disease, degree of proteinuria and HbA1c [50]. Elevated urinary levels of connective tissue growth factor (CTGF) that is activated downstream from

TGF- β_1 were also found in diabetic patients, with micro- and macro-albuminuria [54, 55].

ACE inhibitors and/or angiotensin II receptor blockers (ARBs) reduce the urinary TGF- β_1 excretion rate [33, 26, 55]. Dual renin-angiotensin system (RAS) blockade by ramipril and candesartan in patients with proteinuria >1 g/24 h is followed by decrease of urinary TGF- β_1 and CTGF levels [26, 33, 48, 55, 56].

Renal Transplantation

Elevated urinary TGF- β_1 levels were found during the early post-transplantation period [28]. The extremely high levels of the first few days gradually decrease over a two-month period but remain higher to that of healthy subjects. The increased urinary TGF- β_1 excretion probably represents local production since TGF- β_1 was immunohistochemically identified within the cytoplasm of tubular epithelial cells in patients with delayed graft function [28]. Similar results of elevated TGF- β_1 levels correlated with urinary albumin and α_1 -microglobulin excretion have been reported in renal allograft recipients, 6 months after transplantation [57]. These findings suggest that, proximal tubular epithelial cell injury is involved in the urinary concentration of TGF- β_1 and in the progressive deterioration of renal function after kidney transplantation [57]. Elevated urinary TGF- β_1 levels which were related to the degree of interstitial fibrosis have been also reported in patients with chronic allograft nephropathy [58, 59]. The synthesis and expression of TGF- β_1 in the renal allograft is also induced by a direct effect of cyclosporin-A [60].

Thus, upregulation of renal TGF- β_1 production occurs in the transplanted kidney that is particularly susceptible to ischemia/reperfusion injury as a result of the reintroduction of oxygen free radicals, resulting from anaerobic metabolism [61]. Although restoration of injury and improvement of renal function occurs over the following days, urinary TGF- β_1 excretion remains well above normal. This suggests that a low-grade activation of inflammatory process with production of cytokines and growth factors is present in the graft leading to chronic injury [61, 62].

Conclusions

TGF- β_1 is the main modulator of the healing process and its release ceases when this process has been completed. However, if TGF- β_1 release is not switched off extracellular matrix components are accumulated and tissue

Table 2. Urinary TGF- β_1 levels in renal diseases and transplantation

Type of nephropathy	Urinary TGF- β_1 levels	Relation of urinary TGF- β_1 with		Reduction of urinary TGF- β_1 with	
		proteinuria	histological lesions	immunosuppression	ACEI/ARBs
IgA	high	no	yes	yes	yes
Crescentic	very high ^a	no	yes	no	no data
Membranous	high	yes	yes	yes ^b	no data
Diabetic	high	yes	no data	–	yes
Transplantation	very high ^c	no	yes	no data	no data

ACEI = Angiotensin-converting enzyme inhibitors; ARBs = angiotensin II receptor blockers.

^a Very high levels were mainly observed in patients with crescentic nephritis who showed no improvement of renal function, with immunosuppressive therapy.

^b Reduction of urinary TGF- β_1 levels was observed in patients with remission of nephritic syndrome, with immunosuppressive treatment.

^c Very high levels were observed the first few post-transplant days. Urinary TGF- β_1 levels remain high in renal allograft recipients, especially in those with chronic allograft nephropathy.

fibrosis occurs. Upregulation of TGF- β in the renal tissue of patients with various types of glomerular disease is usually followed by increased urinary TGF- β_1 excretion (table 2) that reflects its intrarenal production. Urinary TGF- β_1 excretion can be accurately determined by sandwich immunoassays using specific antibodies, although commercially available kits or homemade EIAs are, up to now, 'for research use only'.

Elevated urinary TGF- β_1 levels have been reported in patients with acute glomerular inflammation and in renal allograft recipients. The decrease of TGF- β_1 levels in patients with IgA nephropathy with immunosuppressive therapy and in nephrotic patients after remission further supports the implication of this growth factor to the progression of renal disease (table 2). Reduced urinary excretion of TGF- β is also observed with the administration of

ACE inhibitors or ARBs in various types of glomerular disease including diabetic nephropathy. These findings (table 2) suggest that ACE inhibitors or ARBs probably provide renal protection by reducing the renal production of TGF- β_1 .

Hence, urinary TGF- β_1 appears to be a marker of the severity of the glomerular disease and also a marker of the response to treatment towards inhibition of a permanent damage. However, it is still necessary to be elucidated whether a serial determination of urinary TGF- β_1 levels in the same patients could be used as a noninvasive tool to assess the progression of renal disease and to select patients who might need a more intensive treatment. We believe that further research would reveal the importance of producing 'kits for diagnostic use' for the determination of urinary TGF- β_1 .

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