Urinary transforming growth factor-β1 in membranous glomerulonephritis


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Abstract

Background. Human idiopathic membranous glomerulonephritis (MGN) has a highly variable clinical course and factors determining its outcome are poorly known. Since transforming growth factor-β1 (TGF-β1) has an essential role in renal fibrogenesis, we studied the possibility to use urinary excretion of TGF-β1 in the assessment of progression of the disease in patients with MGN.

Methods. Urinary TGF-β1 was determined in 41 patients with MGN, 25 healthy subjects, six non-proteinuric renal transplant patients, 10 patients with IgA glomerulonephritis, and seven proteinuric patients (with non-progressive diseases) using a novel, double antibody enzyme immunoassay. The results were compared with renal morphology and clinical indices of activity of MGN over 12 months.

Results. The median urinary TGF-β1 excretion (pg/mg creatinine) was significantly higher (1730; range 60–16970) in MGN patients than in the healthy controls (300; 30–1330; P < 0.0001). In renal allograft recipients the excretion was 840 (250–3440; P < 0.0001 vs healthy controls), in IgA GN it was 1130 (30–4910; P = 0.039), and in proteinuric patients it was 39 (29–165; P = NS). In MGN but not in the proteinuric controls or renal allograft recipients, urinary TGF-β1 correlated with urinary albumin excretion (r = 0.86, P < 0.0001) but no correlation with renal function or the duration of the disease was found. Urinary TGF-β1 at renal biopsy correlated with interstitial cellular inflammation and its excretion 1 year before the biopsy correlated with indices of sclerosis/fibrosis. Immunosuppressive therapy significantly decreased urinary TGF-β1 from 2800 (1610–16960) to 840 (170–1600) pg/mg creatinine (P = 0.028). Patients with persistent nephrotic syndrome and/or declining renal function had a higher initial TGF-β1 excretion (median 3680; 1830–7420 pg/mg creatinine) than those entering partial or complete remission (1060; 60–1960; P = 0.003) within 12 months from sampling.

Conclusions. Urinary TGF-β1 excretion was increased in patients with MGN, and high excretion indicated intrarenal sclerosing/fibrosing processes and progressive clinical course. Measuring urinary TGF-β1 may be useful in the assessment of the progression of disease and the effects of treatment in MGN.

Key words: growth factors; immunosuppressive therapy; progression; fibrosis

Introduction

A puzzling feature of glomerulonephritides (GN) is the irrevocably progressive course in a considerable proportion of the patients, resulting in end-stage renal failure. In general, the tendency towards progression is primarily related to the morphological type of the glomerular lesion although the evolution may be highly variable even within one category of disease. A typical example is idiopathic membranous glomerulonephritis (MGN) where the clinical course ranges from persistent remission to relapsing disease activity and progressive renal failure [1–3]. Even in the early phases of this disease inflammatory changes may be detected in interstitial areas [4] and progressive disease is characterized by accumulation of extracellular matrix (ECM) components into the mesangial areas, often thickening of the glomerular basement membrane [5] and almost invariably increasing interstitial fibrosis and tubular atrophy [6].

The pathogenesis of glomerular sclerosis and interstitial fibrosis involves action of numerous cytokines and growth factors [7]. The production of these mediators may be triggered either by immunological or non-immunological (metabolic) factors [7]. During the past few years several studies have strongly suggested a unique role for transforming growth factor-β (TGF-β) in the development of sclerosis and fibrosis [8,9]. This peptide, comprising three related dimeric proteins (TGF-β1, 2, and 3), is important in embryogenesis and wound healing, and overexpression may induce immune responses, cellular proliferation, and cytokine
production [9,10]. Importantly, TGF-β increases production of numerous ECM proteins, decreases degradation of ECM and enhances expression of integrins which serve as adhesion receptors for ECM components [8]. Furthermore, inhibition of TGF-β has been shown to attenuate the production of ECM and glomerular inflammation [11,12].

Treatment of MGN is still controversial [13,14] and it should probably be directed to those at high risk of developing progressive renal failure. So far the clinical activity and short-term outcome of MGN have been assessed by protein excretion and glomerular filtration rate [15,16]. Since experimental studies have suggested an important role for mediators of renal inflammation, their urinary excretion has been tested as an indicator of inflammation within the kidneys. Accordingly, urinary IL-6 and IL-6/EGF ratio may indicate activity of the mesangial proliferative GN [17–19] and excretion of terminal complement complexes activity of MGN [20,21]. Recently we reported a possible prognostic ratio of elevated urinary TNF-α concentrations in MGN [22].

Since recent evidence indicates that TGF-β1 plays an essential role in the progression of renal disease, we measured the urinary excretion of this growth factor in patients with MGN by an enzyme immunoassay (EIA). We also present interim results on relationships between urinary TGF-β1 and renal morphology, short-term clinical course and effects of immunosuppressive therapy.

**Subjects and methods**

**Patients with membranous glomerulonephritis**

The study population comprised 41 patients with idiopathic MGN (Table 1) biopsied and followed up at the Helsinki University Central Hospital. In 11 of the patients urinary and serum samples were obtained at the first renal biopsy, in 17 patients at rebiopsy, and in 13 patients with previously diagnosed (biopsied) MGN during an outpatient visit. The median duration of disease (calculated from the first renal biopsy) at sampling was 19 (0–272) months.

**Controls**

The control population consisted of 25 healthy subjects who were studied as outpatients (Table 1). The following patients were included in the study as additional control subjects and studied at renal biopsy: renal transplanted patients (n=6) having no proteinuria six months after the transplantation (protocol biopsies), patients (n=10) with IgA GN having variable degree of proteinuria, and proteinuric patients (n=7) with normal or nearly normal glomerular histology (minimal change GN in five, thin basement membrane disease in one, normal morphology with slight proteinuria in one) and no signs of interstitial fibrosis or progressive renal disease (three patients with minimal-change GN had transient renal failure at sampling) during a follow-up of 12 months.

The study protocol was approved by the local ethical committee and patients and control subjects gave their informed consent.

**Laboratory studies**

**Urinary and serum samples**

Twenty-four-hour urines were obtained, the samples were centrifuged at 3000 r.p.m. for 10 min, and the clear supernatants were stored at −20°C. Venous blood samples were collected in prechilled tubes and centrifuged within 30 min and serum samples were stored at −20°C.

**TGF-β1 assay**

The concentration of TGF-β1 was measured by EIA as follows. Microtitre plates (Maxisorp) were coated with 0.1 μg/well monoclonal mouse IgG-type antibodies to TGF-β1 (Genzyme Co., USA) in 0.05 mol/l Na₂CO₃ buffer, pH 9.0, by incubating overnight at 4°C. The walls were washed with phosphate buffer (0.05 mol/l phosphate, pH 7.3, 0.05% Tween 20). Thereafter 100 μl of acid activated (100 mmol/l HCl, +4°C, for 2 h) and neutralized standard dilutions and undiluted urine samples were incubated in the wells at 4°C overnight. The TGF-β1 bound into the wells was then detected with polyclonal IgG-type sheep antibodies to TGF-β1 (1 ng/well, for 2 h at 37°C) (Jansen Biochemicala, USA) which were labelled with alkaline phosphatase. Alkaline phosphatase activity was determined using p-nitrophenylphosphate as substrate in 1 mol/l diethanolamine-0.5 mol/l MgCl₂ buffer (pH 10, 30 min at 37°C). Natural, human TGF-β1 (R&D Systems, UK) served as standard. Detection limit of the assay was 10 ng/l. The intra-assay and interassay coefficients of variation were 5.9 and 8.1% respectively. The recoveries of added (50 and 100 ng/l) TGF-β1 ranged from 87 to 103%. For calculations TGF-β1 values below the detection limit were assigned as 5 ng/l. To compensate for alterations caused by varying urinary concentration the excretion of TGF-β1 was related to concomitant urinary creatinine (crea).

<table>
<thead>
<tr>
<th>Table 1.</th>
<th>Patients and controls when the first samples for urinary TGF-β1 were obtained</th>
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<tbody>
<tr>
<td>Group</td>
<td>n</td>
</tr>
<tr>
<td>MGN</td>
<td>41</td>
</tr>
<tr>
<td>Renal allografts</td>
<td>6</td>
</tr>
<tr>
<td>Proteinuric controls</td>
<td>7</td>
</tr>
<tr>
<td>IgA GN</td>
<td>10</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>25</td>
</tr>
</tbody>
</table>

Median (range); MGN, membranous glomerulonephritis; IgA GN, IgA glomerulonephritis; ND, not determined.
Changes in urinary TGF-β1 from day to day

To examine the stability of urinary TGF-β1 excretion from day to day, six persons collected 24-h urines over 3 consecutive days.

Other laboratory tests

Renal function was assessed by serum creatinine (normal values from 50 to 130 μmol/l) and GFR (51Cr-EDTA-clearance or 24-hour creatinine clearance, normal value over 80 ml/min/1.73 m²). Serum creatinine, albumin, and creatinine were measured using routine methods.

Renal biopsies

Biopsies, obtained using 14G Bioptry® needles (Manan Medical Products, Northbrook, IL, USA), were processed as described previously [23]. The lesions were classified using an international system [24] and scored throughout the study by the same investigator (TT) who was unaware of the urinary TGF-β1 concentrations. Twenty patients with MGN had their urinary TGF-β1 excretion compared with morphological alterations in simultaneously obtained renal biopsies. In seven biopsied patients with previously diagnosed MGN the morphological alterations were additionally compared with the urinary TGF-β1 values obtained 1 year before the biopsy.

The following morphological alterations were scored semiquantitatively (scale 0, 1, 2, and 3): increased thickness of the glomerular basement membrane, amount of mesangial matrix, tubular atrophy, interstitial fibrosis, interstitial cellular infiltration, and the sum of scores for mesangial matrix and interstitial fibrosis (assigned as sclerosis index).

Statistical methods

Values are expressed as medians (range) unless otherwise indicated. As the distribution of TGF-β1 values, corrected by urinary creatinine content was skew, non-parametric analysis of variance (ANOVA; Kruskall–Wallis) was employed for repeated measurements. Comparisons between two groups were performed with Mann–Whitney U test. The effect of treatment was evaluated with Wilcoxon’s signed rank test. Simple and multiple linear regression analyses were used to study interrelationships between various parameters. Comparison between renal morphology and urinary TGF-β1 was done after logarithmic conversion of the TGF-β1 values. The calculations were performed using Systat® (Systat Inc., USA) and StatView 512+® (BrainPower Inc., USA) softwares, and P values less than 0.05 were considered significant.

Results

Changes in urinary TGF-β1 excretion from day to day

Figure 1 shows the variation of 24-h urinary TGF-β1 excretion (pg/mg crea) in six persons over 3 consecutive days. It appeared that the excretion remained quite constant from day to day.

Urinary TGF-β1 in patients and control subjects

Figure 2 depicts the urinary TGF-β1 excretions. Healthy control subjects had a median TGF-β1 excretion of 300 (30–1330) pg/mg crea (220 ng/l) and patients with MGN an excretion of 1730 (60–16970) pg/mg crea (1200 ng/l) (P < 0.0001 vs normal controls). In patients having stable renal allografts the excretion was 840 (250–3440) pg/mg crea (1700 ng/l) (P < 0.0001), in IgA GN it was 1130 (30–4910) pg/mg crea (970 ng/l) (P = 0.039), and in proteinuric controls...
Urinary TGF-β1 and morphological findings

When urinary TGF-β1 was compared with renal morphology in simultaneously obtained biopsies it appeared that the excretion correlated with the degree of interstitial cellular inflammation but not with other variables (Table 2). However, in seven patients urinary TGF-β1 obtained 12 months before the biopsy correlated significantly with the amount of interstitial fibrosis \((r = 0.86, \text{ P} = 0.01)\) and the sclerosis index \((r = 0.84, \text{ P} = 0.02, \text{ univariate analysis})\).

Immunosuppressive therapy and urinary TGF-β1

Six patients had the urinary TGF-β1 excretion studied before and after immunosuppressive therapy (i.e. plus oral methylprednisolone or cytotoxic drugs), indication for the therapy typically being deteriorating renal function and/or persistent nephrotic syndrome and total duration of treatment varying from 6 to 8 months (Figure 3). Before the therapy the median TGF-β1 excretion was 2800 (1610–16960) pg/mg crea and it decreased during 12 months to 840 (170–1600) pg/mg crea \((\text{P} = 0.028)\). Proteinuria decreased from 13.3 (6.3–27.5) to 7.7 (1.4–12.8) g/24 h \((\text{P} = 0.023)\) and serum creatinine from 176 (83–303) to 143 (71–165) µmol/l \((\text{P} = 0.086)\) during the therapy.

Urinary TGF-β1 and clinical course of MGN

The usefulness of urinary TGF-β1 assay in predicting the short-term clinical course of recently diagnosed MGN was analysed in 14 patients (Figure 4). They all had the initial urinary TGF-β1 measured at or within 12 months of diagnosis (renal biopsy), the nephrotic syndrome (protein excretion exceeding 3 g/24 h), and normal or nearly normal renal function (serum creatinine less than 130 µmol/l, GFR over 60 ml/min/1.73 m²). The patients were divided into two groups according to the clinical status at 12 months after the initial TGF-β1 assay: (a) persistent nephrotic syndrome and/or declining GFR (GFR decreasing below the normal reference value by more than 20%), or (b) remitting disease when the patient entered either partial (urinary protein excretion 0.15–2.9 g/24 h) or complete remission (proteinuria less than 0.15 g/24 h with normal GFR).

In group (a) \((n = 8)\) with a baseline protein excretion of 8.4 (5.6–11.4) g/24 h and age 49 (25–66) years the initial urinary TGF-β1 excretion was 3680 (1830–7420) pg/mg crea, while in group (b) \((n = 6)\) with a protein

<table>
<thead>
<tr>
<th>Alteration</th>
<th>During renal biopsy</th>
<th>One year before renal biopsy</th>
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<tbody>
<tr>
<td></td>
<td>(r)</td>
<td>(P)</td>
</tr>
<tr>
<td>Thickness of GBM</td>
<td>0.32</td>
<td>0.17</td>
</tr>
<tr>
<td>Amount of mesangial matrix</td>
<td>0.02</td>
<td>0.92</td>
</tr>
<tr>
<td>Amount of tubular atrophy</td>
<td>0.27</td>
<td>0.25</td>
</tr>
<tr>
<td>Amount of interstitial fibrosis</td>
<td>0.29</td>
<td>0.22</td>
</tr>
<tr>
<td>Interstitial inflammation</td>
<td>0.52</td>
<td>0.02*</td>
</tr>
<tr>
<td>Sclerosis index</td>
<td>0.16</td>
<td>0.50</td>
</tr>
</tbody>
</table>

In 20 patients urinary samples obtained during renal biopsy and in seven patients samples obtained 1 year before the biopsy are compared with morphological alterations. GBM, glomerular basement membrane; sclerosis index, sum of scores for the amount of mesangial matrix and interstitial fibrosis; NS, not significant. Linear regression analysis. Asterisks indicate significant relationships.
Urinary TGF-β1 excretion and clinical course of membranous glomerulonephritis during 12 months after diagnosis (biopsy). Patients having persistently active, progressive disease (nephrotic syndrome and/or declining renal function) and those entering remission are shown. Short lines indicate median values for the groups (P = 0.003, Mann–Whitney U test).

In these patients with the nephrotic syndrome the clinical status at 12 months correlated with the initial urinary TGF-β1 excretion (r = 0.79, P = 0.002) but not with the baseline proteinuria (r = 0.1, P = NS, univariate analysis). Also in a multiple regression analysis where the clinical status at 12 months was the dependent variable, the short-term clinical course correlated highly significantly with the initial urinary TGF-β1 (P = 0.004) but not with the baseline proteinuria or age.

Discussion

The present study focuses on MGN, a prototype of an immune-mediated, slowly progressive glomerulonephritis in which the clinical and morphological course is unpredictable [2,25]. It appeared that the majority of patients had an elevated urinary TGF-β1, highly increased excretion being associated with a progressive renal disease.

Urinary TGF-β excretion has previously been studied only in some experimental and clinical settings. Rabbit crescentic nephritis, induced by glomerular basement membrane antibodies, was associated with an increased TGF-β excretion which peaked 7 days after the induction of the disease and correlated with its severity [26]. In humans the urinary TGF-β excretion was significantly increased in focal segmental glomerulosclerosis only, slightly elevated in systemic lupus erythematosus but not in IgA nephropathy or MGN (where it was actually decreased) [27]. Renal allograft recipients studied by Coupes et al. [28] had elevated plasma levels of TGF-β1 but their urinary TGF-β1 excretion was comparable with the normal subjects as it also was in MGN patients.

Our findings may differ from the above human studies for several reasons. The results of Kanai et al. [27] are not directly comparable because many patients received prednisolone treatment and possibly had rather inactive diseases (as judged from minimal proteinuria). TGF-β1 molecules, synthesized as precursors are cleaved to monomers and associated with the latency associated protein [9]. Activation of this latent complex is caused by acidification, alkalinization, heating, or proteases [9,29], and in previous studies the methods for activation of urinary TGF-β1 have been variable (if done at all). Finally, differences in the antibodies and standards used in assays may explain the disparities between the studies.

Urinary TGF-β1 correlated with albumin excretion in MGN (and IgA GN as well) whereas proteinuric patients with normal renal histology (and a non-progressive disease) had low TGF-β1 excretion and no correlation with albuminuria. On the other hand, non-proteinuric renal allograft recipients had elevated TGF-β1 excretion. These findings suggests that the elevated TGF-β1 excretion in MGN is not simply explained by an increased glomerular permeability of proteins although this issue warrants further studies.

Proteinuria reflects the immunological activity (i.e., the size and location of electron-dense deposits) of MGN [25] and its quantity is a rough indicator of the severity and prognosis of this disease [2]. It is possible that active MGN leading to and toxic effects of long standing proteinuria on mesangial and tubular cells [30] are related to increased urinary TGF-β1 in MGN. It is also theoretically possible that reduced absorption of filtered TGF-β1 may have contributed to the elevated urinary values.

We did not study the production of TGF-β1 in the renal tissue. However, experimental and human studies have demonstrated that renal injury is associated with an increased expression of TGF-β by glomerular mesangial [12,29,31] and epithelial cells [32] as well as by the tubulointerstitial compartment [33]. In adriamycin nephropathy interstitial inflammatory cells express TGF-β1 [34]. Platelets contain high concentrations of TGF-β [7,8] but their depletion does not modulate renal injury in experimental glomerulonephritis [8]. Monocytes may not only produce but also express receptors for TGF-β1 whereby it can affect monocytic infiltration and their secretion of TNF-α and IL-1 [35]. Thus our findings together with above studies strongly suggest that the most probable sources of urinary TGF-β1 are renal parenchymal and/or invading cells.

In the present study there was a relationship between urinary TGF-β1 and interstitial inflammation which may point to a role for inflammation as a trigger of TGF-β1 synthesis. However, a single TGF-β1 value may not reliably reflect the duration of inflammation
(i.e. whether inflammatory activity was temporary or prolonged) before sampling which may determine the severity of resulting sclerosing phenomena. This may explain why no correlation with sclerosis or interstitial fibrosis was found. However, TGF-β1 excretion studied 1 year before the renal biopsy, theoretically reflecting exposure of renal tissue to the effects of TGF-β1 after sampling, correlated with biopsy indices of sclerosis/fibrosis. Furthermore immunosuppressive therapy significantly decreased TGF-β1 excretion in MGN. The above findings are in concord with the previous experimental study [26] and suggest that urinary TGF-β1 reflects ongoing intrarenal accumulation of ECM proteins and thus sclerosing/fibrosing processes which may be affected by treatment.

A characteristic feature of MGN is its highly variable clinical course [1–3,16]. The prediction of the outcome of the disease is difficult but long-lasting abundant proteinuria and/or decreasing GFR [15,16] together with abundant tubulointerstitial alterations [6] are considered the most reliable indicators of poor prognosis. Since immunosuppressive therapy may improve the prognosis in MGN [13,14] it would be highly desirable if this potentially toxic therapy could be directed to those at risk of progressive renal disease whilst these drugs should be avoided in those who will remit. Our findings suggest that urinary TGF-β1 could be used to predict the short-term outcome of MGN. Longitudinal studies are still needed to elucidate whether serial determination of urinary TGF-β1 is useful in assessing the outcome of MGN.

In conclusion, urinary TGF-β1 excretion was found to be elevated in MGN. Persistently high TGF-β1 excretion correlated with morphological indices of chronicity. In addition, highly increased excretion suggested persistently active and/or progressive clinical course, whereas less elevated values suggested normal GFR and remission. Furthermore immunosuppressive treatment significantly decreased urinary TGF-β1. Presuming that urinary TGF-β1 reflects ongoing sclerosing and fibrosing processes in the kidneys, its determination could be used as a non-invasive tool to assess the progression of renal disease, to select MGN patients for immunosuppressive therapy, and to follow-up of the effects of treatments aimed at suppressing the production of TGF-β1.

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References


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