Serum and urinary transforming growth factor beta 1 as biochemical markers in diabetic nephropathy patients

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Abstract

Diabetic nephropathy is a common complication of diabetes mellitus. Transforming growth factor beta 1 (TGF-β1) is considered to be one of the major cytokines involved in the regulation of extracellular matrix (ECM) synthesis and degradation. Discrepant results were reported for urine TGF-β1 in diabetic patients. The aim of the present study is to investigate urine and serum TGF-β1 in patients with type II diabetes with and without nephropathy.

The study was performed on 72 patients with type II diabetes (26 macroalbuminuria, 27 microalbuminuria and 19 normoalbuminuria) together with 30 healthy subjects to serve as controls. Urinary and serum TGF-β1 and urine albumin were investigated by Elisa, plasma glucose, whole blood glycated hemoglobin, Urinary and serum total protein, creatinine, were determined by colorimetric methods. Urine and serum TGF-β1 were significantly increased in all diabetic groups being more pronounced in the macroalbuminuria group than micro and normoalbuminuria groups. Also urine total protein was increased, being more pronounced in macroalbuminuria group than other groups. Urinary and serum TGF-β1 showed high positive correlation with urinary total protein concentration in macro and microalbuminuria groups r = (0.9, 0.9, 0.8 and 0.89) respectively. The results revealed significant increase in Urinary and serum TGF-β1 and their concentrations in urine were parallel to urine proteins and albumin concentrations; urinary and serum TGF-β1 showed high positive correlation with urinary total protein, so it could be used as a marker with total protein and albumin to confirm the diabetic nephropathy in type II diabetic patients.

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1. Introduction

Diabetes mellitus is a metabolic disease, which is characterized by high glucose levels in blood (hyperglycemia) and urine (glucosuria). Diabetes affects more than 170 million people worldwide and the number will rise to 370 million people by 2030. About one third of those affected, will eventually have progressive deterioration of renal function (Chen et al., 2013). A long standing diabetic state of diabetes mellitus can result in several severe chronic complications: cardiovascular complications, diabetic retinopathy, diabetic neuropathy and diabetic nephropathy (Ramezani et al., 2007). Diabetic nephropathy (DN) is one of the most severe complications of diabetes mellitus (El Mesallamy et al., 2008). The classical definition of diabetic nephropathy is a progressive rise in urine albumin excretion, coupled with increasing blood pressure, leading to declining glomerular filtration and eventually end stage kidney failure (Obineche and Adem, 2005). Diabetic nephropathy is characterized structurally by renal hypertrophy and by progressive mesangial deposition of extracellular matrix (ECM) characterizing glomerulosclerosis, which is associated with progressive glomerular capillary occlusion, albuminuria and a progressive fall in glomerular filtration rate (GFR). Overt diabetic nephropathy is characterized by persistent proteinuria (>500 mg/24 h) or macroalbuminuria (>300 mg/24 h) (Rivarola et al., 1999). In the natural history of the disease, proteinuria is preceded by stages of excessive glomerular filtration and of microalbuminuria (30–300 mg/24 h), which signals an increased risk of progression to overt nephropathy (Williams, 2005). The degree of proteinuria correlates with the progression of glomerulosclerosis and tubulointerstitial fibrosis (Wolf and Ziyadeh, 2007).

Transforming growth factor beta (TGF-β1) is a multifunctional cytokine implicated in the pathogenesis of many forms of progressive renal disease, including diabetic nephropathy (McKnight et al., 2007). TGF-β1 directly stimulates the transcription of many extracellular matrix genes in renal cells including mesangial, endothelial and tubular cells. In addition, it decreases collagenase production and simultaneously stimulates expression of tissue inhibitors of metalloproteinases, resulting in inhibition of extracellular matrix turnover. Deposition of extracellular matrix components, including fibronectin and collagen types I, III and IV, is an important component of the scarring observed during the progression of glomerulosclerosis and tubulointerstitial fibrosis. An increase in the synthesis as well as a decrease in turnover of these proteins is responsible for the net accumulation of extracellular matrix (Fukuda et al., 2009).

A number of molecular mediators and intracellular signaling pathways that have been identified in diabetic kidney injury have been found to stimulate the renal TGF-β1 activity as an intermediary step. These mediators are: high glucose concentration, early and advanced products of nonenzymatic glycation of proteins, oxidative stress, glomerular hypertension, de novo synthesis of diacylglycerol and protein kinase C activation, glucosamine overproduction, and high levels of vasoactive substances such as intrarenal angiotensin II, endothelin, and thromboxane (Ziyadeh, 2004). Several clinical studies have established that TGF-β1 is increased in the kidneys of diabetic patients (Sharma and McGowan, 2000). Urinary TGF-β1 measurement has been suggested as a marker for diabetic nephropathy in some studies as reported by Sato et al. (1998) who found higher TGF-β1 excretion in diabetic patients well correlated to the state of nephropathy, other studies have not shown the association of urinary TGF-β1 with diabetic nephropathy as reported by Eljia et al. (2000) who did not find difference in urinary TGF-β1 excretion between microalbuminuric and normoalbuminuric patients.

The aim of the present work is to study the role of TGF-β1 as biochemical marker in diabetic nephropathy which was monitored by glycemic status and kidney function measurements and to explore the correlation between serum TGF-β1 and urinary TGF-β1 in diabetic nephropathy although it has been suggested as a marker for diabetic nephropathy not all studies had shown the association of urinary TGF-β1 with nephropathy.

2. Patients and methods

2.1. Patients

This study included 102 subjects which classified into 2 main groups.

Group (I): thirty adult hypertensive healthy volunteers served as control. They were on antihypertensive therapy with captopril. The control group was selected as hypertensive healthy subjects since all the diabetic patients in group II were hypertensive. The systolic and diastolic BP values for all subjects were shown in Table 1.

Group (II): seventy two previously diagnosed type 2 diabetic patients from outpatients’ clinic of the National Institute for Urology and Nephrology, Cairo, Egypt. All type 2 diabetic patients met the criteria of American Diabetes Association (ADA) for type 2 diabetes. All type 2 diabetic subjects were not receiving any medications other than hypoglycemic drugs and hypotensive drug (Captopril) and were not complaining of any chronic or acute illness.

This group was subdivided into the following subgroups according to albumin excretion rate (AER):

- Group IIa nineteen diabetic patients with normoalbuminuria having (AER<30 mg/g creatinine).
- Group IIb Twenty seven diabetic patients with microalbuminuria having (AER 30–300 mg/g creatinine).
- Group IIc Twenty six diabetic patients with macroalbuminuria having (AER > 300 mg/g creatinine).

Full history including age, sex, diabetic duration and treatment were recorded for all subjects. The studied groups were matched as regards age and sex. Written informed consent was obtained from the subjects and this study was approved by the Ethics Committee of the National Research Center.

2.2. Exclusion criteria

Chronic liver diseases, heart diseases, coronary artery diseases (CAD), malignancy, autoimmune diseases, chronic renal
Table 1 – Demographic and bio-clinical characteristics of healthy control subjects (group I) and diabetic patients (groups IIa, IIb and IIc).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I</th>
<th>Group IIa</th>
<th>Group IIb</th>
<th>Group IIc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>30</td>
<td>19</td>
<td>27</td>
<td>26</td>
</tr>
<tr>
<td>Age (years)</td>
<td>48.2 ± 8.1</td>
<td>54.3 ± 6.9</td>
<td>55.8 ± 7.9</td>
<td>55.5 ± 4.9</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>18/12</td>
<td>10/9</td>
<td>18/9</td>
<td>12/14</td>
</tr>
<tr>
<td>DM duration (years)</td>
<td>–</td>
<td>9.3 ± 6.5</td>
<td>10.4 ± 5.2</td>
<td>13.6 ± 5.7bc*</td>
</tr>
<tr>
<td>No. of patients receiving Insulin/Oral therapy</td>
<td>–</td>
<td>7/12</td>
<td>14/13</td>
<td>17/9</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>132.3 ± 6.2</td>
<td>133.1 ± 8.2</td>
<td>132.5 ± 11.2</td>
<td>132.3 ± 11.7</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>80.6 ± 6.9</td>
<td>80.5 ± 9.1</td>
<td>79.6 ± 7.5</td>
<td>80.7 ± 10.1</td>
</tr>
<tr>
<td>Fasting plasma glucose (mg/dl)</td>
<td>88.7 ± 11.3</td>
<td>177.6 ± 60.3</td>
<td>194.2 ± 52****</td>
<td>198.1 ± 57.8****</td>
</tr>
<tr>
<td>Glycated hemoglobin (%)</td>
<td>7.2 ± 0.5</td>
<td>9.3 ± 1.4</td>
<td>11.6 ± 2.6**,b,*</td>
<td>10.1 ± 1.9***,c,*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD, a: significant difference from healthy group (group I), b: significant difference from diabetic normoalbuminuria (group IIa), c: significant difference from diabetic microalbuminuria (group IIb). *: P < 0.05, **: P < 0.01, ***: P < 0.001.

2.3. Samples collection and biochemical analysis

Blood samples were drawn in the morning after a 12 h fast (from 8 pm to 8 am); a portion of the blood was collected on EDTA for the determination of glycated hemoglobin. The other portion left to clot and serum was separated for the other determinations. Morning urine samples were collected, the urine was centrifuged at 1000 rpm, and the supernatant was used for biochemical analysis.

2.4. Methods

Fasting plasma glucose was determined by enzymatic colorimetric method performed according to Trinder reaction using a Kit provided by linear chemicals (Barcelona-Spain). Glycated hemoglobin was measured in whole blood chromatographically and colorimetrically using a kit obtained from intermedical (Italy). Serum and urine creatinine were measured colorimetrically by the reaction of creatinine in serum or urine with picric acid in alkaline condition to form a yellow-orange color complex (Jaffe reaction), using a kit obtained from Bio-MED diagnostics (Egypt). Urine total proteins were measured colorimetrically using a kit obtained from Linear chemicals (Barcelona-Spain). Albuminuria was determined by ELISA using a kit provided by DRG Diagnostics (USA) according to the method of Walker et al. (1992). Urine and serum TGF-β1 was measured by ELISA using a kit provided by RayBiotech (USA) according to the method of Kropf et al. (1997).

2.5. Statistical analysis

All statistical analyses were performed using the SPSS version 17. The data were expressed as Mean ± standard deviation (SD). Individual groups were compared using Student T-test and different groups were compared using analysis of variance (ANOVA) followed by Bonferroni post hoc test to compare individual groups. Moreover, correlations between different parameters were evaluated by Pearson’s correlation (r), Receiver operator characteristic (ROC)-curve analysis was used to establish the cut-off values for TGF-β1.

3. Results

In diabetic groups, the diabetic patients with macroalbuminuria showed a significant long duration of diabetes in comparison with micro-albuminuria and normo-albuminuria (P < 0.05). Fasting plasma glucose and glycated hemoglobin were significantly increased in macroalbuminuria and microalbuminuria in comparison with healthy control group (P < 0.001). On the other hand, in macroalbuminuria group there was an elevation in fasting plasma glucose in comparison with microalbuminuria and normoalbuminuria groups (Table 1).

The macroalbuminuria group and the microalbuminuria group showed highly significant increase in urinary total protein concentration and urinary albumin concentration in comparison with both normoalbuminuria and healthy control groups (P < 0.001). As regards urinary creatinine the macroalbuminuria group showed highly significant increase in comparison with the normoalbuminuria group (P < 0.001), also the microalbuminuria group showed a significant decrease in comparison with the normoalbuminuria group (P < 0.01), while there is no statistical significant difference between the macroalbuminuria and the healthy control group (Table 2). The high concentrations of urinary creatinine were in the normoalbuminuria and the healthy control groups and the low concentration were in the macroalbuminuria and microalbuminuria. Albumin/creatinine ratio and total protein/creatinine ratio showed significant increase in macroalbuminuria and microalbuminuria when compared with normoalbuminuria and healthy control groups (P < 0.001). Regarding serum total protein, the healthy control and normoalbuminuria groups showed highly significant increase in comparison with macroalbuminuria group (P < 0.001). Being the largest concentration of serum total protein was in the healthy control group followed by the normoalbuminuria then the microalbuminuria groups, and the least concentration was in the macroalbuminuria group. Serum creatinine concentration showed significant increase in macroalbuminuria in comparison with both microalbuminuria and...
Urinary and serum TGF-β1 showed weak correlation with duration of diabetes mellitus in macro, micro and normalalbuminuria groups $r = (0.2, 0.3, 0.3, 0.2, 0.2$ and 0.4) respectively.

Urine and serum TGF-β1 denoting high positive correlation with urinary total protein concentration in macro, micro and normalalbuminuria groups $r = (0.9, 0.9, 0.7, 0.8, 0.89$ and 0.73) respectively, also high positive correlation with urinary albumin concentration in macro, micro and normalalbuminuria groups $r = (0.87, 0.78, 0.81, 0.8, 0.84$ and 0.79) respectively, while no and weak correlation with healthy control group.

Significant positive correlations were observed between both urinary and serum TGF-β1 and proteinuria/creatinine ratio in diabetic nephropathy patients $r = (0.76$ and 0.77) respectively (Fig. 5). Also, significant positive correlations were detected between both urinary and serum TGF-β1 and albuminuria/creatinine ratio in diabetic nephropathy patients $r = (0.62$ and 0.64) respectively (Fig. 6). While there were no correlations in diabetic nonnephropathy patients and healthy control subjects.

Urine and serum TGF-β1 showed a positive correlation with urinary creatinine in macro, micro and normalalbuminuria groups $r = (0.49, 0.49, 0.6, 0.37, 0.47$ and 0.5) respectively, but with a negative correlation in healthy control group.

### Table 2 – Biochemical data of healthy control subjects (group I) and diabetic patients (groups IIa, IIb and IIc).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Gp I</th>
<th>Gp IIa</th>
<th>Gp IIb</th>
<th>Gp IIc</th>
</tr>
</thead>
<tbody>
<tr>
<td>U. Total protein (mg/l)</td>
<td>Mean ± SD</td>
<td>63.9 ± 13.6</td>
<td>148.2 ± 52</td>
<td>403.8 ± 158&lt;sup&gt;b, c&lt;/sup&gt;</td>
</tr>
<tr>
<td>U. Creatinine (g/l)</td>
<td>Mean ± SD</td>
<td>1.2 ± 0.6</td>
<td>1.7 ± 0.6</td>
<td>1 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>U. Total protein/Creatinine (mg/g)</td>
<td>Mean ± SD</td>
<td>63.9 ± 35.5</td>
<td>92.8 ± 27.8</td>
<td>472.4 ± 212&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>U. Albumin (mg/l)</td>
<td>Mean ± SD</td>
<td>6.6 ± 1.4</td>
<td>35 ± 15.1</td>
<td>126.3 ± 45.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>U. Albumin/Creatinine (mg/g)</td>
<td>Mean ± SD</td>
<td>7 ± 4.5</td>
<td>21 ± 6</td>
<td>151.2 ± 74.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>S. Total protein (g/l)</td>
<td>Mean ± SD</td>
<td>71.2 ± 7.6</td>
<td>71 ± 6.8</td>
<td>67.7 ± 5.5</td>
</tr>
<tr>
<td>S. Creatinine (mg/dl)</td>
<td>Mean ± SD</td>
<td>0.9 ± 0.2</td>
<td>1.02 ± 0.1</td>
<td>0.9 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD, a: significant difference from healthy group (group I), b: significant difference from diabetic normoalbuminuria (group IIa), c: significant difference from diabetic microalbuminuria (group IIb). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

### Table 3 – Urine and serum TGF-β1 data of healthy control subjects (group I) and diabetic patients (groups IIa, IIb and IIc).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Gp I</th>
<th>Gp IIa</th>
<th>Gp IIb</th>
<th>Gp IIc</th>
</tr>
</thead>
<tbody>
<tr>
<td>U. TGF-β1 (pg/g creatinine)</td>
<td>Mean ± SD</td>
<td>8 ± 3.7</td>
<td>13.1 ± 6.6</td>
<td>31.7 ± 18.6&lt;sup&gt;b, c&lt;/sup&gt;</td>
</tr>
<tr>
<td>S. TGF-β1 (pg/ml)</td>
<td>Mean ± SD</td>
<td>16.2 ± 6</td>
<td>23.3 ± 15.9</td>
<td>201 ± 79.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a: significant difference from healthy group (group I), b: significant difference from diabetic normoalbuminuria (group IIa), c: significant difference from diabetic microalbuminuria (group IIb). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

Fig. 1 – Correlation between urinary TGF-β1 and Plasma glucose in diabetic patients with macroalbuminuria (a) and microalbuminuria (b).
Urinary and serum TGF-β1 showed no correlation to weak correlation with serum creatinine ($r = 0.002 \sim 0.224$) in macro, micro and normoalbuminuria groups and with no correlation with the healthy control group.

Urinary TGF-β1 showed high positive correlation with serum TGF-β1 ($r = 0.86, 0.93$ and $0.89$) in macro, micro and normoalbuminuria groups respectively but with weak correlation in healthy control group ($r = 0.269$).

**Fig. 2** — Correlation between serum TGF-β1 and Plasma glucose in diabetic patients with macroalbuminuria (a) and microalbuminuria (b).

**Fig. 3** — Correlation between urinary TGF-β1 and glycated hemoglobin in diabetic patients with macroalbuminuria (a) and microalbuminuria (b).

**Fig. 4** — Correlation between serum TGF-β1 and glycated hemoglobin in diabetic patients with macroalbuminuria (a) and microalbuminuria (b).
The sensitivity, specificity and accuracy were 88.7%, 98% and 0.931 respectively as regards urinary TGF-β1 (cutoff point was 20.9). While the sensitivity, specificity and accuracy were 100%, 73.5 and 0.872 respectively as regards serum TGF-β1 (cutoff point was 25.15) (Fig. 7).

4. Discussion

The present study was designed to evaluate the value of TGF-β1 as a biochemical marker for diabetic nephropathy in type II diabetic patients. The present work showed that urinary and serum TGF-β1 levels were significantly increased in diabetic macro and microalbuminuria groups when compared with healthy control subjects and that was confirmed by the positive significant correlations between both glucose concentration and glycated hemoglobin and both urinary and serum TGF-β1, and these results agreed with Hellmich et al. (2000), Hefini et al. (2007) and El Mesallamy et al. (2012). TGF-β1 concentration increased in diabetic patients with albuminuria whether macro or microalbuminuria (diabetic patients with nephropathy) not with diabetic normoalbuminuria (diabetic patients without nephropathy) or control subject who were hypertensive, these results were supported by Yaqiu et al. (2001) who concluded that the serum concentration of TGF-β1 has started to increase in the early stages of diabetic nephropathy and with the development of diabetic nephropathy the serum level of TGF-β1 significantly increased.

Urinary and serum TGF-β1 showed correlation with duration of diabetes. The mean duration of diabetes is greater in macroalbuminuria when compared with both normoalbuminuria and microalbuminuria groups. These results are in agreement with El Mesallamy et al. (2012) who found that diabetic patient with microalbuminuria and macroalbuminuria showed a significant long duration of diabetes in comparison with normoalbuminuria.

Significant positive correlations were found between the concentration of TGF-β1 in urine and both proteinuria and albuminuria in patients with type II diabetes, these results agreed with Rivarola et al. (1999) and Hefini et al. (2007), however there were wide variations in the rate of urinary and serum TGF-β1 concentrations in these patients which may be due to that the patients were presented with different stages of nephropathy. Several studies have shown that TGF-β1 may play a major role in glomerular disease mediating the inflammatory response through glomerulosclerosis (Coimbra et al., 1991; Border et al., 1992, Yamamoto et al., 1993; Bertoluci et al., 1996), but there are some observations...
suggesting that urinary TGF-β1 derives from renal biosynthesis and not from ultrafiltration or secretions (Noh et al., 1993; Dominguez et al., 1998; Grainger et al., 1995). Also these results may coincide with the result that urinary and serum TGF-β1 were negatively correlated with the serum total protein in macro, micro and normoalbuminuria groups with no significant correlation with the health control subjects, and this may denote loss of big amount of protein in urine hence its scanty in serum of diabetic patients.

The present study showed low mean concentration of urinary creatinine in the macro and microalbuminuria groups and relatively high mean concentration of urinary creatinine in the macroalbuminuria and healthy control groups. In the present study high levels of serum creatinine were detected in the macroalbuminuria group, while lower levels were detected in the healthy and microalbuminuria groups. These variability in the results of urinary and serum creatinine may be attributed to the occurrence of low glomerular filtration rate, which result from renal hypertrophy, progressive mesangial deposition of extracellular matrix (ECM) and progressive glomerular capillary occlusion. The factors responsible for the deposition and accumulation of extracellular matrix material within the kidney are hyperglycemia, glycated proteins, vasoactive hormones, systemic and glomerular hypertension, proteinuria, growth factors, and cytokines which have been implicated in the pathogenesis of diabetic nephropathy (Reeves and Andreoli, 2000).

5. Conclusion

It is well known fact that the onset of type II diabetes is insidious, and by time of diagnosis many patients may develop overt nephropathy, hence the present study recommends screening for urinary and/or serum TGF-β1 concentration as soon as diabetes is firstly diagnosed with regular follow up screening for TGF-β1 afterwards. Thus, this may be considered as biochemical marker that can be used to estimate the progression of diabetes to diabetic nephropathy.

Fig. 7 – ROC curve of urinary (a) and serum (b) TGF-β1.

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