Isoelectric focusing of urinary transferrin for detection of early stages of diabetic nephropathy

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SUMMARY

Urinary albumin (ALB) and transferrin (TF) are useful markers for impaired renal function. Compared to ALB, TF is more readily excreted into urine from an early stage of renal failure because of its smaller number of negative charges. However, few evidence of a change in isoelectric point (pI) value of urinary TF in nephropathy patients has been reported. We analyzed pI values of urinary TF by an isoelectric focusing, and compared the results in normal subjects with those in diabetic patients at various stages of nephropathy. Urine samples of diabetics were randomly collected from 24 patients and 7 healthy volunteers. An apotransferrin with a high pI value was detected in samples of healthy subjects. However, the pI value of TF bands derived from diabetic patients shifted to the lower side, and the more nephropathy stages progressed, greater change of pI value was detected. In addition, TF with low iron-binding capacity was detected in some samples from diabetics. These results suggest that determination of the pI value of urinary TF in diabetic patients may be useful diagnostic markers for an early detection of diabetic nephropathy.

Key words: pre-nephropathy, early nephropathy, isoelectric point, iron saturation of transferrin, glycation of transferrin

INTRODUCTION

A frequent complication of diabetes is diabetic nephropathy, which often requires the patient to undergo extracorporeal dialysis. However, early diagnosis and treatment can prevent diabetic patients from suffering chronic renal failure. For this reason many nephropathy markers, such as albumin (ALB), transferrin (TF), N-acetylglucosamine and type IV collagen, have been reported. ALB and TF are present in the urine of healthy subjects at very low levels, but are easily detected in the urine of nephropathy patients. Therefore, urinary ALB and TF are useful markers for diabetic nephropathy1−5. However, Caramori et al. reported that microalbuminuria did not reflect progression of nephropathy and expansion of mesangial proliferation6.

In diabetic nephropathy, urinary TF excretion is elevated earlier than ALB excretion. Thus, TF has potential as a diagnostic marker for the early stage of nephropathy7−9. A decrease in anionic sites in the glomerular basement membrane was reported to be one cause of proteinuria10,11. Hence, TF having less negative charge than ALB is more readily excreted into the urine of patients with early diabetic nephropathy. Furthermore, elevated excretion of ALB into urine in diabetic nephropathy can be caused by defective renal tubular reabsorption of ALB or by dysfunction of a barrier protein, such as nephrin in the glome-rulus12,13.

TF is a serum glycoprotein of the β-globulin fraction comprising a single polypeptide with a molecular mass of 77 kDa. Human TF has two N-linked oligosaccharide chains in the C-terminal domain, and the number of sialic acid units at the terminus of the oligosaccharide chain of TF can vary14. Furthermore, one TF molecule can bind two iron atom at each N- and C-terminal domain, and three isoforms of TF can exist with depending on the iron binding status; non-iron binding TF, monoferric TF, diferric TF. Differences in terms of the number of sialic acid units or iron content affect the isoelectric point (pI) of TF. Thus, many isoforms of TF can exist with a wide range of pI

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Analysis of urinary TF is thought to be useful for the detection of renal dysfunction at an early stage. However, few evidence concerning changes in the pI value of TF in diabetic nephropathy patients has been reported. Therefore, a study of the molecular diversity of TF from urine samples of diabetic nephropathy patients is needed to evaluate the usefulness of urinary TF analysis for nephropathy.

In this study, we analyzed urinary TF in diabetics using Isoelectric focusing (IEF) and immunoblotting. By comparing the results obtained from healthy subjects and diabetics we were able to correlate changes in the molecular diversity of TF with the onset of diabetic nephropathy. The likely mechanism for the increase in urinary TF during the early stages of diabetic nephropathy is also discussed. Finally, the potential use of urinary TF as a marker for the diagnosis of diabetic nephropathy is evaluated.

Materials and Methods

Samples

Urine samples were collected from 24 diabetic patients who underwent clinical examinations at Sanraku Hospital. Normal spot urine and plasma were collected from 7 healthy volunteers in the Tokyo Medical and Dental University. All samples were stored at –20°C prior to analysis.

Analysis of urine components

Urine components were quantified by Clinical Analyzer 7070 (Hitachi Ltd., Tokyo, Japan) using commercially available kits as follows: urinary ALB; Micro-ALB (Nittobo Medical Co., Tokyo, Japan), urinary TF; Micro-TF (Nittobo Medical Co.), urinary creatinine (Cre); Determiner CRE (Kyowa Medex Co., Tokyo, Japan).

Modifications of TF molecules

TF was saturated with iron (III) according to the method reported by Lot et al.18 Six times volume of a sample was added to a solution of 0.1 g/mL iron (III) chloride (Wako Pure Chemicals Ind., Osaka, Japan) and incubated for 5 min at room temperature.

Glycation of TF protein was performed as follows. 0.1 mL of glucose solution (100 g/L) was added to 0.9 mL of control serum (Consera, Nissui Pharmaceutical Co., Tokyo, Japan), and filtered through a 0.22 μm pore size membrane. The solution was then incubated for 14 days at 37°C.

TF microheterogeneity analysis

IEF was performed using a cellulose acetate membrane (CA membrane, Separax SP, Fujifilm Co., Tokyo, Japan) and electrophoretic instrumentation (CoolPhoreStar, Anatech, Tokyo, Japan). The conditions for IEF were according to our reported method with slight modification19. The 30 min preliminary electrophoresis at 500 V was followed by sample application and stepwise electrophoresis as follows: 300 V for 10 min, 500 V for 30 min, 1500 V for 45 min and 2000 V for 20 min. One to five μL of urine samples were applied to a CA membrane according to the TF concentration. We calculated the pI of each band by using the isoelectric focusing calibration kit (GE Healthcare, Amersham, UK).

Immunoblotting was performed as reported previously20. After IEF, the protein in the CA membrane was transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore Co., Bedford, MA) by overlaying the CA membrane with a PVDF membrane for 18 hr. After blocking with blocking solution (Blockace, Dainippon Pharmaceutical Co., Osaka, Japan), TF was detected with rabbit anti-human transferrin antibody (Dako Norden A/S, Glostrup, Denmark), anti-rabbit IgG (H+L) horseradish peroxidase conjugate (Bio-Rad Laboratories, Inc., Hercules, CA). Finally, protein bands were visualized using diaminobenzidine tetrahydrochloride (WAKO Pure Chemical Industries, Osaka, Japan) or ECL Western Blotting Detection System (GE Healthcare, Amersham, UK).

Analysis for molecular weight of serum TF

The molecular mass of serum and urinary TF was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide gel21. The electrophoresed protein was transferred onto PVDF membrane using a transfer module (AE-6656, Atto Co., Tokyo, Japan) at a constant current of 2.0 mA/cm² for 45 min. TF was detected by the same method as described above.

RESULTS

Determination of urinary ALB and TF at various stages of Diabetic nephropathy

The stages of diabetic nephropathy were classified by dividing urinary ALB by urinary Cre as follows: pre-nephropathy (under 30 mg/g-Cre; normo-albuminuria), early nephropathy (between 30 and 300 mg/g-Cre; micro-albuminuria) and overt nephropathy (over 300 mg/g-Cre; macro-albuminuria)22. As shown in Fig. 1, the TF level correlated with the determined ALB level, and increased by progression of diabetic nephropathy except two cases of early and overt nephropathy. The concentration of TF in all diabetic patients was much higher than that of healthy subjects: 2.5 times in pre-nephropathy, 23 times in early nephropathy, and 178 times in overt nephropathy stages on average (Table 1).

Analysis of serum and urinary TF in healthy subjects

To elucidate the difference in pI values and molecular weight of serum and urinary TF, samples were analyzed by IEF and SDS-PAGE. In the result of IEF, a pI range of serum TF was different from that of urinary TF. The range of serum TF in sample of normal subject was around pI 5.00 and 5.60, and the main band of TF frequently appeared...
around pI 5.25 (Fig. 2(A)). Urinary TF bands from healthy subjects were detected in the range pI 5.20 to 5.60. When samples in TF were treated with iron (III) chloride prior to IEF analysis, the pI values of both serum and urinary TF were significantly affected i.e. the bands converged at lower pIs. The pI value of the main band was about 5.10 (Fig. 2(B)). The molecular mass of TF analyzed determined by SDS-PAGE was 80 kDa in both serum and urine samples (Fig. 2(C)).

Analysis of urinary TF from patients at various stages of nephropathy

Urinary TF derived from each patient with nephropathy was analyzed by IEF prior to iron saturation. As shown in Fig. 3, the pI value of the main band of TF gave a progressively lower value according to the progress of nephropathy in the early stages of the disease. Samples taken from patients in early nephropathy showed similar results to subjects in pre-nephropathy or in overt nephropathy. The pattern for TF taken from patients with overt nephropathy the IEF gave a similar pattern to serum samples of healthy subjects. Table 2 shows the range of TF bands: TF in the range pI 5.12–5.61 for patients in pre-nephropathy stage; pI 5.05–5.55 for patients in early stage of nephropathy; pI 5.20–5.70 for diabetics in overt nephropathy stage.

To examine whether the state status of iron saturation of TF affects mobility during IEF, urine samples were treated with iron prior to analysis. After iron saturation, the TF bands corresponding to TF moved to the anode side: TF in pI range 4.94–5.44 for the pre-nephropathy stage; pI 4.85–5.45 for the early stage of nephropathy; pI 4.90–5.40 for diabetics in the overt nephropathy stage (Table 3). A lot of samples derived from patients in the early and overt stages of nephropathy were significantly affected by the addition of iron: i.e. TF bands converged at a lower pI. However, some samples of pre- and early nephropathy stage were not responsive to iron saturation (Fig. 4).

Table 1. Concentration of ALB and TF in the samples of each nephropathy stage.

| Criteria     | Healthy subjects (mean±SD) | Diabetes (mean±SD) |
|--------------|----------------------------|---------------------|
|              | N 7                        | pre-nephropathy     | early nephropathy   | overt nephropathy |
| ALB (mg/g • Cre) | 8.4±4.6                   | 11.4±6.6           | 114.3±73.9          | 1196.5±1601.4*   |
| TF (mg/g • Cre)  | 0.4±0.3                    | 1.0±0.6            | 9.0±0.6             | 71.1±15.0*       |

Overt nephropathy stage significantly differed from pre-nephropathy stage assessed by one way ANOVA with Tukey’s HSD method (P<0.05).

Fig. 1. Correlation between urinary TF and ALB at each stage of nephropathy.

Closed circles, closed triangles and closed squares correspond to the pre-nephropathy stage, early nephropathy stage and overt nephropathy stage, respectively.

Fig. 2. Electrophoretic profile in IEF and SDS-PAGE of TF for urine (U) and serum (S) samples from healthy subject.

(A) IEF pattern of TF without iron treatment; (B) IEF pattern of TF treated with iron; (C) SDS-PAGE. TFs were detected by anti-human transferrin antibody on PVDF membranes transferred from CA membrane (IEF) or polyacrylamide gel (SDS-PAGE).
Effect of glycation on the pI value of TF

To elucidate the mechanism of the observed shift of TF to the anode side from patients with diabetes, serum samples were treated with glucose prior to IEF. After glycation, the ratio of serum TF with a lower pI increased, the pI range of TF detected by IEF shifted to anode side (Fig. 5).

DISCUSSION

The excretion of ALB and TF into urine of diabetic patients with nephropathy is thought to be due to increased permeability of the glomerular basement membrane caused by a reduction of heparin sulfate during hyperglycemia. TF, having negative charge less than ALB, is more readily excreted during nephropathy. Therefore, TF was reported to be a useful marker of impaired renal function.\(^{7-9}\) It was also reported that the mechanism for urinary excretion of TF can be explained by the decrease of ability of the renal proximal tubules to reabsorb TF.\(^{23}\) That is, a decrease in the expression of megalin in epithelial cells of the proximal renal tubule was reported to cause defective renal reabsorption of ALB or TF.\(^{24-26}\) Furthermore, urinary ALB, which is used for the diagnosis of diabetic nephropathy, was reported to have no correlation with expansion of the mesangium region.\(^{6}\) Thus, urinary TF may behave in a similar way to ALB.

In this study, we examined the relationship between urinary ALB and TF. Here, we reveal that urinary ALB and TF are well correlated with the progression of nephropathy, except in cases where ALB showed an elevated value (Fig. 1). Our results suggest that the excretion mechanism of TF might be the same as that of ALB in conditions of advanced nephropathy. While an ALB amount excreted into urine in healthy subjects was similar to that in pre-nephropathy subjects, TF excretion during pre-nephropathy increased 2.5-fold from healthy subjects. This result is similar to those obtained in the study by Kanauchi et al.\(^{27}\) and this finding suggests that changes in the excretion amount of TF might reflect renal abnormalities at an early stage.

The property of TF derived from urine was compared to that from serum. The molecular weight of TF from both samples was same, but the pI values were different; in healthy subjects urinary TF had less negative charge than TF from serum samples (Fig. 2). Thus, the negative charge of the protein appears to affect excretion into the urine.

### Table 2. The pI range of TF in the samples of each nephropathy stage and healthy subject without iron saturation.

| Criteria            | pI range of TF (mean±SD) |
|---------------------|--------------------------|
|                     | N  | anode side | cathode side |
| healthy subject     | 7  | 5.19±0.04  | 5.61±0.11    |
| pre-nephropathy     | 8  | 5.12±0.08  | 5.61±0.06    |
| early nephropathy   | 9  | 5.05±0.22  | 5.55±0.13    |
| overt nephropathy   | 7  | 5.20±0.07  | 5.70±0.07\(^*\) |

\(^*\) Overt nephropathy stage significantly differed from healthy subject assessed by one way ANOVA with Tukey's HSD method (P<0.05).

### Table 3. The pI range of TF in the samples of each nephropathy stage and healthy subject after iron saturation.

| Criteria            | pI range of TF (mean±SD) |
|---------------------|--------------------------|
|                     | N  | anode side | cathode side |
| healthy subject     | 7  | 5.09±0.16  | 5.51±0.16    |
| pre-nephropathy     | 8  | 4.94±0.05  | 5.44±0.17    |
| early nephropathy   | 9  | 4.85±0.20\(^*\) | 5.45±0.16    |
| overt nephropathy   | 7  | 4.90±0.10\(^**\) | 5.40±0.10    |

\(^*\) Early nephropathy stage significantly differed from healthy subject assessed by one way ANOVA with Tukey's HSD method (P<0.01).

\(^**\) Overt nephropathy stage significantly differed from early nephropathy stage assessed by one way ANOVA with Tukey's HSD method (P<0.05).
When the urine samples were saturated with iron, the same IEF pattern was obtained as that of serum TF. These observations indicate that a substantial proportion of TF in the urine sample of healthy subjects exist as apotransferrin.

Sialic acid is substituted at the terminus of the sugar chain bound to TF. Elimination of sialic acid during circulation in the blood would reduce the overall negative charge of TF. Furthermore, the pI value of TF changes according to the iron binding status of the protein; pI 5.2 for diferric TF, pI 5.6 for monoferric TF and pI 6.1 for non iron-bound TF. Among the various isoforms of TF, urinary TF is more negatively charged in accordance with the progression of nephropathy (Fig. 3). Notably, the detected range of urinary TF in pre-nephropathy subjects shifted slightly to the anode side of the IEF pattern. The main band of TF shifted to a lower pI value according to the degree of nephropathy. These results suggest that changes in renal function are detectable even in the early stages of nephropathy. IEF of the early nephropathy samples divided the samples into 2 groups according to the behavior of the main band. In one group, the position of the main band was the same as that in the pre-nephropathy sample; in the other group, the position of the band was the same as in the overt nephropathy sample (Fig. 3). Thus, it is possible to diagnose the stage of nephropathy by analyzing the molecular diversity of TF. Such diagnosis by IEF analysis of TF cannot be derived from quantifying the amount of urinary protein alone.

We investigated the necessity of iron saturation in IEF analysis for evaluation of TF as a valuable marker of nephropathy. After saturation with iron, TF bands in some samples from diabetic subjects were detected in a lower pI range than those of the samples from healthy subjects (Table 3, Fig. 2 and Fig. 4). Some samples of early or overt nephropathy were unaffected by treatment with iron (Fig. 4, lanes 1, 2 and 4). The binding capacity of TF for iron is likely to be pH-dependent. However, pH of the various urine samples was identical (data not shown). Because the iron-binding capacity of TF is known to decrease as the glycation of the protein is increased, IEF after iron saturation might be affected by the glycation status. We conclude that iron saturation treatment is unnecessary prior to IEF analysis of urinary TF in diabetic nephropathy.

When serum TF was treated with glucose, the negative charge of TF increased (Fig. 5). We presume that the anode shift in the pI range of TF in early nephropathy from that in overt nephropathy could be caused by increased glycation. The IEF pattern of TF for urine samples of overt nephropathy was similar to that obtained from serum samples. This might be caused by a loss of function in the renal barrier for protein filtration and leakage of a large amount of serum TF into the urine.

Stibler et al. investigated the relationship between the structure of the sugar chain of TF and alcohol intake. They reported that the sialic acid content of TF was reduced in serum samples from alcoholic subjects and that in such samples TF with a higher pI was frequently detected by IEF analysis. Urinary TF is derived from the serum component, which could be affected by alcohol intake or glycation to change pI values of TF. However, the altered TFs account for only a small fraction of serum TF. Therefore, alcohol intake or glycation is unlikely to greatly affect the detection of a TF band shift by IEF.

Although glomerular mesangium expansion is quite important among pathogenic changes in the early stages of nephropathy, the expansion of mesangium was not correlated with albuminuria. Therefore, new markers for detecting mesangium expansion or glomerular sclerosis are needed. Our results clearly demonstrated that IEF analysis of urinary TF can be used as an early diagnostic marker for diabetic nephropathy. Further studies to investigate the possible mechanism of TF excretion to urine are required.

**ABBREVIATIONS**

ALB, albumin; TF, transferrin; pI, isoelectric point; IEF, isoelectric focusing; Cre, creatinine; PVDF, polyvinylidene fluoride
mide gel electrophoresis; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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