Significance of Urinary Type IV Collagen in Patients With Diabetic Nephropathy Using a Highly Sensitive One-Step Sandwich Enzyme Immunoassay

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Urinary concentrations of type IV collagen in patients with diabetic nephropathy were measured by a highly sensitive, one-step sandwich enzyme immunoassay. Samples from 298 patients with non-insulin-dependent diabetes mellitus (NIDDM) and 80 healthy controls were examined. In diabetic patients with macroalbuminuria or renal insufficiency, the concentrations of urinary type IV collagen were significantly higher than those of diabetic patients with normoalbuminuria or healthy controls ($P < 0.001$). Urinary type IV collagen concentration in diabetic patients with microalbuminuria was signifi-

cantly higher than that in diabetic patients with normoalbuminuria or that in healthy controls ($P < 0.001$). In contrast, there were no significant changes in the concentration of serum type IV collagen between microalbuminuric patients and normoalbuminuric patients. The area under the receiver operating characteristic (ROC) curve for the urinary type IV collagen concentration was equivalent to that of urinary albumin. It was concluded that urinary type IV collagen concentration determined using this method might be a useful marker for the early detection of diabetic nephropathy. *J. Clin. Lab. Anal.* 11:110–116. © 1997 Wiley-Liss, Inc.

Key words: diabetic nephropathy; non insulin-dependent diabetes mellitus (NIDDM); type IV collagen; receiver operating characteristic analysis; microalbuminuria

INTRODUCTION

Diabetic nephropathy is one of the major complications of noninsulin-dependent diabetes mellitus (NIDDM) as well as insulin-dependent diabetes mellitus (IDDM) and one of the main causes of increased morbidity and mortality in such patients. Pathologic alterations of diabetic nephropathy include the progressive expansion of glomerular mesangial matrix and thickening of the glomerular basement membrane (GBM) (1–4). Type IV collagen is the major collagenous component of GBM and the mesangial matrix and exists as a triple helix of $\alpha(IV)$ chains with a noncollagenous (NC) globular domain at its carboxy terminus. It has been reported that serum and urinary levels of type IV collagen in diabetic nephropathy are increased compared to those in diabetic patients without diabetic nephropathy or in control subjects (5–7).

The aim of the present study was to measure the serum and urinary concentrations of type IV collagen using a highly sensitive, one-step sandwich enzyme immunoassay (EIA) and to investigate whether these parameters might be useful clinical markers for the early detection of diabetic nephropathy.

MATERIALS AND METHODS

Patients

A total of 298 patients (193 males, 105 females) with NIDDM and 80 healthy controls (49 males, 31 females) were examined for measurement of serum and urinary type IV collagen. Diabetic patients were diagnosed using the criteria of the Japan Diabetic Society (8). Patients who had liver diseases or such systemic diseases as rheumatoid arthritis or systemic lupus erythematosus were excluded from this study. Diabetic patients ranged in age from 23 to 81 (55.6 ± 10.7) years, and the healthy controls ranged from 22 to 68 (44.8 ± 13.5) years of age (Table 1). Retinopathy was classified in 117 diabetic patients as none (76 patients), simple (15 patients), preproliferative (eight patients), or proliferative (18 patients) retinopathy determined by skillful ophthalmologists.

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TABLE 1. Clinical Characteristics of Controls and Diabetic Patients

	Control	Stage I	Stage II	Stage III	Stage IV
n	80	127	98	45	28
Age (y)	44.8 (13.5)	55.2 (9.9) ^a	54.7 (11.2) ^a	55.3 (11.6) ^a	60.1 (10.7) ^{a,d,g}
Sex (Male:Female)	49:31	81:46	59:39	31:14	22:06
BMI (kg/m ²)	—	23.6 (3.5)	23.4 (3.6)	23.7 (4.0)	24.0 (3.6)
Duration of diabetes (y)	—	8.1 (6.2)	10.9 (7.2) ^c	10.6 (7.2) ^c	13.8 (7.7) ^b
MBP (mmHg)	—	104.3 (11.9)	104.7 (11.6)	112.5 (13.3) ^{c,f}	110.6 (13.8)
FPG (mM)	—	9.00 (3.08)	10.23 (4.35) ^d	9.78 (3.74)	8.18 (2.12) ^g
HbA1c (%)	—	8.2 (1.9)	8.8 (1.6) ^d	8.8 (1.9)	7.9 (1.2) ^{f,i}
BUN (mM)	—	5.37 (1.27)	5.90 (2.20) ^d	5.79 (1.74)	11.32 (5.72) ^{b,e,h}
s-Cr (μM)	—	54.6 (17.6)	54.6 (16.6)	61.2 (14.7) ^{d,g}	153.7 (78.0) ^{b,e,h}
Retinopathy		(n=57)	(n=31)	(n=16)	(n=13)
no		49	18	7	2
simple		5	5	3	2
preproliferative		1	4	3	0
proliferative		2	4	3	9

Mean (1 SD)

^a*P*<0.001 vs control.^b*P*<0.001.^c*P*<0.01.^d*P*<0.05 vs. Stage I.^e*P*<0.001.^f*P*<0.01.^g*P*<0.05 vs. Stage II.^h*P*<0.001.ⁱ*P*<0.05 vs. Stage III.

Reagents

Bovine serum albumin (BSA) (Fraction V) was obtained from Sigma Chemical Company (St. Louis, MO). Pepsin from porcine gastric mucosa and horseradish peroxidase (HRP, Grade 1) were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Polystyrene balls (6.5 mm in diameter) were obtained from Precision Plastic Ball Co. (Chicago, IL). N-(ε-maleimidocaproyloxy) succinimide (EMCS), and 3,3',5,5'-tetramethylbenzidine (TMBZ) were obtained from Dojindo Lab (Kumamoto, Japan). Protein A-Cellulofine was obtained from Seikagaku Kogyo (Tokyo, Japan); 2-aminoethanethiol-HCl and other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of Human Type IV Collagen and Monoclonal Antibodies

Type IV collagen was isolated by salt precipitation from pepsin digests of human placental tissues according to the method of Mayne et al. (9) with some modification. Mouse monoclonal antibodies against pepsin-solubilized type IV collagen were prepared as described (10). Antibodies (IgG1) in ascites from hybridoma clones 4H12 and 1D3 were fractionated by 40% saturated ammonium sulfate followed by centrifugation at 8,000g for 20 min. The former, clone No. 4H12, was characterized by the specificity to the 7S domain, and the latter, clone No. 1D3, was characterized by the specificity to the triple helix (TH) domain of type IV collagen.

The precipitates were dialyzed in 1.5 M glycine-NaOH buffer, containing 0.5 M NaCl (pH 8.9), and then the solution containing the antibodies was applied to a protein A-Cellulofine column, which was previously equilibrated with the same dialysis buffer as described above. IgG1 antibodies were eluted with 0.1 M Na-citrate buffer (pH 5.0). Purification of antigen and antibodies were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Baum et al. (11).

Preparation of Fab'-peroxidase Conjugate

Purified mouse monoclonal anti-TH domain antibody (clone No. 1D3) was digested with pepsin from porcine mucosa to obtain F(ab')₂. The F(ab')₂ were reduced with 2-aminoethanethiol, and the Fab'-peroxidase conjugate was prepared by the method of Hashida et al. (12) using EMCS as a maleimide compound for the conjugation of Fab' to HRP through thiol groups in the hinge region.

Preparation of Monoclonal Antibody-coated Polystyrene Balls

Polystyrene balls were coated by physical adsorption. The procedure was performed according to the method of Ishikawa et al. (13) at 4°C for 24 hours with 0.1 g/l purified mouse monoclonal anti-7S domain antibody (clone No. 4H12) in 0.1 M Na-phosphate buffer (pH 7.5) containing 0.1% Na₃N. The balls were washed five times with 10 mM Na-phos-

phate buffer (pH 7.0) containing 0.1% BSA, 0.1 M NaCl. The balls were stored at 4°C and washed in 10 mM Na-phosphate buffer (pH 7.0) containing 0.1% BSA and 0.1 M NaCl just before use.

One-Step Sandwich EIA Technique

A 100 µl aliquot of standard pepsin-solubilized type IV collagen or urine was mixed with 300 µl of mouse monoclonal anti-TH domain antibody, Fab'-HRP conjugate (0.8 mg/l) in 0.01 M Na-phosphate buffer (pH 7.0) containing 0.5% BSA, 1.5% (v/v) horse serum, 0.01% geneticin, and 0.1 M NaCl in a plastic test tube. A polystyrene ball coated with mouse monoclonal anti-7S domain antibody was then added to the solution. Each of the plastic test tubes was left for 24 hours at 8°C for immunoreaction. After that, the polystyrene ball was washed twice with 3.5 ml 0.01 M Na-phosphate buffer (pH 7.0) containing 0.1 M NaCl and then transferred to another new plastic test tube, which was incubated with 300 µl of TMBZ (0.134 g/l) and 100 µl of hydrogen peroxide (0.15 g/l) for 1 hour at 25°C by the method of Bos et al. (14). The reaction was stopped by adding 1 ml sulfuric acid (0.67 M) and the absorbance at 450 nanometers was measured by a Micro-Flow spectrophotometer (Shimadzu, Model UV730, Kyoto, Japan).

Analysis

Measurement of urinary type IV collagen concentrations was performed by the above-mentioned highly sensitive, one-step sandwich enzyme immunoassay (EIA) with two monoclonal antibodies to the 7S domain and the TH domain (Fuji Chemical Industries, Co., Toyama, Japan). Serum type IV collagen concentrations were measured by EIA. Measurement of urinary transferrin (Tf) or N-acetyl β-D-glucosaminidase (NAG) concentration was performed by the latex turbidimetric immunoassay (LTIA) and substrates for the NAG assay (MCP-NAG method).

Proteinuria was detected by N-Multistix SG-L® (Bayer-Sankyo, Co., Tokyo, Japan). Urinary albumin:creatinine (Cr) ratio was measured for the early detection of diabetic nephropathy in patients without proteinuria. Urinary albumin concentration was measured by turbidimetric immunoassay (TIA) and the urinary albumin:Cr ratio was calculated from the urinary albumin concentration divided by urinary Cr concentration. Urinary concentrations of type IV collagen and Tf were also divided by the urinary Cr concentration to exclude influence of the urinary volume. The normal values for serum and urinary type IV collagen are < 140 µg l⁻¹ or < 0.47 µg mmol⁻¹, respectively. Based on albuminuria or proteinuria and the levels of serum creatinine (s-Cr), the diabetic patients were divided into four stages as follows (Table 1):

Stage I: Normoalbuminuria (urinary albumin:Cr ratio < 2.0 mg mmol⁻¹)

Stage II: Microalbuminuria (urinary albumin:Cr ratio > 2.0 mg mmol⁻¹)

Stage III: Macroalbuminuria (proteinuria but s-Cr level < 90.0 µmol l⁻¹)

Stage IV: Renal insufficiency (proteinuria and s-Cr level > 90.0 µmol l⁻¹)

A total of 118 diabetic patients and 46 healthy controls were also examined to measure the levels of urinary Tf and NAG.

Clinical Activity

The following examinations and laboratory tests were performed at the time of measurement of serum and urinary type IV collagen: body mass index (BMI), duration of diabetes, mean blood pressure (MBP), fasting plasma glucose (FPG), glycosylated hemoglobin (HbA1c), blood urea nitrogen (BUN) and serum creatinine (s-Cr). HbA1c was measured by high-performance liquid chromatography and normal values ranged from 5.2 to 6.7%.

Statistical Analysis

The concentrations of urinary Tf and NAG were changed to logarithms so that they followed normal distribution. Statistical significance was evaluated by the Student t-test to analyze the differences in the serum and urinary concentration of type IV collagen, logarithms of Tf (log(Tf)) and that of NAG (log(NAG)). Linear regression analysis was used to compare each clinical parameter. We also used the χ^2 analysis to determine the interrelationship of nominal variables. The area under the receiver operating characteristic (ROC) analysis (15, 16) was used to compare the usefulness among urinary concentrations of type IV collagen, albumin, log(Tf) and log(NAG). Data are presented as mean values (1 SD).

RESULTS

Urinary Concentration of Type IV Collagen

Urinary type IV collagen was detected in 54 out of 127 patients (42.5%) in stage I (normoalbuminuria), 66 out of 98 patients (68.3%) in stage II (microalbuminuria), 44 out of 45 patients (97.8%) in stage III (macroalbuminuria), and all 28 patients (100%) in stage IV (renal insufficiency). In contrast, it was detected in only three out of the 80 healthy adults (3.8%). The incidences of detectable urinary type IV collagen in stages III and IV were significantly higher than those in stages I and II and healthy controls (for stage III: $\chi^2 = 41.388$, $P < 0.001$; $\chi^2 = 16.088$, $P < 0.001$; $\chi^2 = 108.526$, $P < 0.001$, for stage IV: $\chi^2 = 30.423$, $P < 0.001$; $\chi^2 = 12.255$, $P < 0.001$; $\chi^2 = 93.890$, $P < 0.001$). The incidence in stage II was significantly higher than that in stage I and healthy controls ($\chi^2 = 13.699$, $P < 0.001$; $\chi^2 = 72.392$, $P < 0.001$). The incidence in stage I was also significantly higher than that in healthy controls ($\chi^2 = 36.973$, $P < 0.001$).

The concentrations of urinary type IV collagen in stage IV were significantly higher than those in stages I, II, and III and in healthy adults ($P < 0.001$, $P < 0.001$, $P < 0.05$, $P < 0.001$,

respectively) (Fig. 1, Table 2). The concentrations of urinary type IV collagen in stage III were significantly higher than those in stage I and II and healthy controls ($P < 0.001$). The levels of urinary type IV collagen concentration in stage II were significantly higher than those in stage I and healthy controls ($P < 0.001$). The urinary type IV collagen concentration in stage I was also significantly higher than that in healthy controls ($P < 0.01$). Significant positive correlations were observed between the concentration of urinary type IV collagen and that of urinary albumin, log(Tf) and log(NAG) ($r^2 = 0.479$, $P < 0.001$; $r^2 = 0.282$, $P < 0.001$; $r^2 = 0.227$, $P < 0.001$, respectively). No correlation was observed between the levels of urinary type IV collagen and actual age, sex, BMI, MBP, or HbA1c. There was a significant positive correlation between the urinary type IV collagen concentration and the duration of diabetes ($r^2 = 0.032$, $P < 0.05$).

Serum Concentration of Type IV Collagen

Type IV collagen in sera was detected in 9 out of 108 patients (8.3%) in stage I, 23 out of 92 patients (25.0%) in stage II, 12 out of 37 patients (32.4%) in stage III, and 18 out of 28 patients (64.3%) in stage IV. However, it was detected in only 2 of the 50 healthy controls (4.0%). The incidence of detectable serum type IV collagen in stage IV was significantly higher than that in stages I, II, and III and healthy controls ($\chi^2 = 43.749$, $P < 0.001$; $\chi^2 = 14.729$, $P < 0.001$; $\chi^2 = 6.507$, $P < 0.05$; $\chi^2 = 34.213$, $P < 0.001$, respectively). The incidence in stage III was significantly higher than in stages I and healthy controls ($\chi^2 = 12.923$, $P < 0.001$; $\chi^2 = 12.731$, $P < 0.001$). The incidence in stage II was also significantly higher than in stage

I and healthy controls ($\chi^2 = 10.268$, $P < 0.001$; $\chi^2 = 9.848$, $P < 0.001$).

The levels of serum type IV collagen concentration in stage IV were significantly higher than those in stages I, II and III ($P < 0.001$, $P < 0.001$, $P < 0.05$). The levels of serum type IV collagen concentration in healthy controls were significantly lower than those in stages III and IV ($P < 0.01$, $P < 0.001$). There was no significant difference in serum type IV collagen levels between stage I and stage II (Table 2).

ROC Analysis of Urinary Type IV Collagen

Areas under the ROC curve for urinary concentrations of type IV collagen, albumin, Tf, and NAG in diabetic patients were 0.78, 0.84, 0.76, and 0.71, respectively. There were no significant differences among these results because of the smaller number of patients ($n = 46$) (Fig. 2). Areas under ROC curve for urinary concentration of type IV collagen and albumin in diabetic patients in stages I and II ($n = 204$) were 0.74 and 0.81; the difference was not significant (Fig. 3).

Relationship to Diabetic Retinopathy

Serum concentration of type IV collagen in patients with proliferative retinopathy was significantly higher than that in patients without retinopathy or healthy controls ($P < 0.001$). Urinary concentrations of type IV collagen in patients with any grade of retinopathy and also without retinopathy were significantly higher than those in healthy controls ($P < 0.001$). The levels of type IV collagen in patients with proliferative retinopathy was significantly higher than that in patients without retinopathy ($P < 0.05$) (Table 3).

DISCUSSION

The present study showed that the concentration of urinary type IV collagen in diabetic patients with microalbuminuria was significantly higher than that in such patients with normoalbuminuria or in healthy controls, and the area under the ROC curve of urinary type IV collagen concentration was equivalent to that of the urinary albumin concentration. The classification of diabetic patients into four stages in this study was decided in accordance with the diagnostic criteria for diabetic nephropathy of the Ministry of Health and Welfare, Japan (17).

The glomerular basement membrane (GBM) represents the size- and charge-selective barrier of the glomerulus. It is composed of a backbone of type IV collagen, which forms the lamina densa, flanked on either side by layers with different compositions, the laminae rara interna and externa. Type IV collagen is also the major collagenous component of the extracellular matrix (ECM). It was generally considered that the accumulation of ECM including type IV and VI collagens, fibronectin and laminin, led to glomerular sclerosis in various kinds of renal diseases (18–23). It has been reported that a high glucose concentration and/or advanced glycation end

Fig. 1. Concentration of urinary type IV collagen in diabetic patients and controls.

TABLE 2. Serum and Urinary Type IV Collagen, Urinary Transferrin, and NAG in Diabetic Patients and Healthy Controls

	Serum type IV collagen ($\mu\text{g/l}$)	Urinary type IV collagen: Cr ratio ($\mu\text{g mmol}^{-1}$)	Urinary Tf:Cr ratio ($\log[\text{mg mmol}^{-1}]$)	Urinary NAG ($\log[\text{U/l}]$)
Control	98 (23)	0.29 (0.09)	-1.54 (0.30)	0.40 (0.43)
Stage I	100 (34)	0.42 (0.37) ^b	-1.41 (0.52)	0.55 (0.38)
Stage II	115 (48)	0.79 (0.61) ^{a,c}	-0.69 (0.50) ^{a,c}	0.68 (0.39) ^b
Stage III	134 (66) ^{b,d}	1.26 (0.70) ^{a,c,e}	0.451 (0.69) ^{a,c}	0.91 (0.31) ^{a,c}
Stage IV	161 (51) ^{a,c,e}	2.85 (2.07) ^{a,c,e,g}	0.90 (0.45) ^{a,c,e,g}	1.09 (0.21) ^{a,c,f}

Mean (1 SD)

^a $P < 0.001$.^b $P < 0.01$ vs. control.^c $P < 0.001$.^d $P < 0.01$ vs. Stage I.^e $P < 0.001$.^f $P < 0.01$ vs. Stage II.^g $P < 0.05$ vs. Stage III.

product (AGE) administration increase the production and expression of type IV collagen in cultured glomerular cells, mainly mesangial cells, and in animal models (24–34). Other studies have shown an increase in the intensity of immuno-histochemical staining for type IV collagen in renal tissues from patients with diabetic nephropathy (18, 23, 35–37). Recently, we reported that mRNA of the synthesis and degradation enzymes of type IV collagen, matrix metalloproteinase-3 (MMP-3), and tissue inhibitor of metalloproteinase-1 (TIMP-1), were expressed by glomerular cells and interstitial cells in situ hybridization of renal tissues from patients with diabetic nephropathy (38).

The sensitivity of previous EIA for the measurement of urinary type IV collagen concentration was so low that urine samples had to be concentrated 20 times before measurement. However, the newly developed EIA used in this study had high sensitivity. This method is characterized by the use of two kinds of monoclonal antibodies against 7S and TH domain of type IV collagen and measurements without any treatment before use. The concentration of urinary type IV collagen in diabetic patients with microalbuminuria was significantly higher than that in those with normoalbuminuria, but there was no significant correlation between the levels of serum type IV collagen in such patients with microalbuminuria and

Fig. 2. ROC analyses and areas under the ROC curves for urinary concentration of type IV collagen (●), albumin (○), transferrin (+), and NAG (△). **Fig. 3.** ROC analyses and areas under the ROC curves for urinary concentration of type IV collagen (●) and albumin (○) in patients with normo- and microalbuminuria (Stages I and II).

TABLE 3. Serum and Urinary Type IV Collagen, Urinary Transferrin, and NAG in Diabetic Patients With or Without Retinopathy and Healthy Controls

	n	Serum type IV collagen ($\mu\text{g l}^{-1}$)	Type IV collagen: Cr ratio ($\mu\text{g mmol}^{-1}$)	Urinary Tf:Cr ratio ($\log[\text{mg mmol}^{-1}]$)	Urinary NAG ($\log[\text{U l}^{-1}]$)
Control	80	98 (23)	0.29 (0.09)	-1.54 (0.30)	0.40 (0.43)
Retinopathy					
no	76	94 (36)	0.68 (0.72) ^a	-1.00 (0.90)	0.60 (0.41)
simple	15	118 (73)	0.89 (0.86) ^a	-0.75 (1.32)	0.67 (0.47)
preproliferative	8	103 (29)	1.07 (0.89) ^a	-0.15 (0.97) ^{a,c}	0.91 (0.32)
proliferative	18	140 (53) ^{a,b}	1.51 (1.27) ^{a,c}	0.05 (1.09) ^{a,b}	0.96 (0.22) ^{a,c}

Mean (1 SD)

^a $P < 0.001$ vs. control.^b $P < 0.001$.^c $P < 0.05$ vs. no retinopathy.

normoalbuminuria. These findings suggested that renal pathological alterations might be reflected in urinary type IV collagen concentration rather than in serum concentration.

Receiver operating characteristic (ROC) analysis is one of the scales to compare usefulness among several clinical examinations (15, 16). If a clinical examination has a better discriminating ability, the ROC curve moves to the upper-left corner and the area under the ROC curve becomes larger. Because the area under the ROC curve for the urinary concentration of type IV collagen and albumin were almost the same in diabetic patients with normo- and microalbuminuria, it was assumed that the levels of urinary type IV collagen had a discriminating capacity for the early detection of diabetic nephropathy equivalent to that of urinary albumin concentration. Further prospective studies on the clinical significance of this test for the early diagnosis of progressive diabetic nephropathy are warranted.

We concluded that highly sensitive, one-step EIA is useful for measurement of type IV collagen in urine from patients with diabetes mellitus, and urinary type IV concentrations may be useful for the early detection of diabetic nephropathy.

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