Asian Multicenter Trials on Urinary Type IV Collagen in Patients With Diabetic Nephropathy

Yasuhiko Tomino,^{1*} Shigenobu Suzuki,¹ Chieko Azushima,¹ Ichiyu Shou,¹ Toshihiko Iijima,² Mitsunori Yagame,³ Li Ning Wang,⁴ Hung-Chun Chen,⁵ Kar-Neng Lai,⁶ Si Yen Tan,⁷ and Myung Jae Kim⁸

¹Division of Nephrology, Department of Medicine, Juntendo University, Tokyo, Japan

²Department of Internal Medicine, Juntendo Urayasu Hospital, Urayasu, Japan

³Division of Nephrology and Metabolism, Department of Internal Medicine, School of Medicine, Tokai University, Isehara, Japan

⁴Division of Nephrology, China Medical University, Shenyang, PROC

⁵Division of Nephrology, Kaohsiung Medical University, Kaohsiung, Taiwan, PROC

⁶Department of Medicine, The University of Hong Kong, Hong Kong, PROC

⁷Division of Nephrology, University of Malaya, Kuala Lumpur, Malaysia

⁸Department of Internal Medicine, Kyung Hee University, Seoul, Korea

To investigate the changes of renal type IV collagen turnover in diabetic nephropathy, urinary type IV collagen was measured by a highly sensitive one-step sandwich enzyme immunoassay (EIA). Urinary samples were obtained from 698 diabetic patients and 191 healthy adults. Among the patients, 264 had urinary albumin levels of less than 29 mg/g-creatinine (Cr) (Stage I: normoalbuminuric stage), 169 had microalbuminuria from 30 to 299 mg/g-Cr (Stage II: microalbuminuric stage), 84 patients had macroalbuminuria of more than 300 mg/g-Cr and serum Cr of less than 1.1 mg/dl (Stage IIIA: macroalbuminuric stage without renal dysfunction), 97 had macroalbuminuria of more than 300 mg/g-Cr and serum Cr of more

than 1.2 mg/dl (Stage IIIB: macroalbuminuric stage with renal dysfunction), and 84 had renal failure (Stage IV). The levels of urinary type IV collagen in Stages II, IIIA, IIIB, and IV were significantly higher than those in Stage I ($P < 0.0001$). The level of urinary type IV collagen in Stage I ($5.00 \pm 0.23 \mu\text{g/g-Cr}$; mean $\pm$ SE) was also higher than that in normal adults ($3.44 \pm 0.11 \mu\text{g/g-Cr}$; mean $\pm$ SE). These levels increased gradually due to progression of the clinical stage of diabetic nephropathy. It appears that the levels of urinary type IV collagen can be a useful marker for detecting renal injuries in diabetes according to our Asian multicenter trials. *J. Clin. Lab. Anal.* 15:188–192, 2001. © 2001 Wiley-Liss, Inc.

Key words: urinary type IV collagen; diabetic nephropathy; Asian multicenter trials

Diabetic nephropathy is one of the major long-term microvascular complications occurring in nearly 40% of diabetic patients in Japan (1). Microalbuminuria is the only clinical sign of late-developing diabetic nephropathy. It is also known that significant structural changes have already appeared even at the stage of microalbuminuria. Thus, it is necessary to develop more sensitive measurements for detecting the early stage of glomerular injury in patients with diabetic nephropathy.

Since type IV collagen is the principal component of glomerular basement membrane (GBM) and mesangial matrix, the levels of type IV collagen in sera and urinary samples may reflect the rate of matrix turnover in diseased kidneys (2–5). It is generally considered that the accumulation of extracellular matrix (ECM) might be regulated by the balance of its

production and degradation by matrix proteinases. In the previous study, we reported an increase of urinary type IV collagen excretion in patients with diabetic nephropathy although no difference in the serum type IV collagen levels was observed (6,7). Recently, Takizawa et al. (8) reported an increase of urinary type IV collagen in normoalbuminuric patients with impaired glucose tolerance (IGT). Thus, it is postulated that an increase of urinary type IV collagen excretion indicates on-going alteration in the turnover of glomerular and tubular type IV collagen in the diabetic kidney.

*Correspondence to: Yasuhiko Tomino, Division of Nephrology, Department of Medicine, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. E-mail: yasu@med.juntendo.ac.jp

Received 18 September 2000; Accepted 29 January 2001

To date, no multicenter studies on the measurement of type IV collagen in diabetic patients have been reported. The purpose of the present study was to determine the relationship between the levels of urinary type IV collagen and the clinical stages of diabetic nephropathy in the first multicenter trials in Asian countries.

MATERIALS AND METHODS

Urinary Samples

Urinary samples were obtained from 698 type I and II diabetic patients and 191 healthy adults used as controls. Diagnosis of type II diabetes and diabetic nephropathy was performed according to the criteria of the Japanese Diabetic Research Council, and Ministry of Health and Welfare, Japan (9,10). None of the patients had any history of liver disease or systemic disorders such as rheumatoid arthritis or systemic lupus erythematosus (SLE). Since all the subjects were outpatients, spot urine was used for the detection of albuminuria and corrected as a ratio of urinary creatinine concentration to avoid the influence of urinary volume.

Among the patients, 264 patients had urinary albumin levels of less than 29 mg/g-creatinine (Cr) (Stage I: normoalbuminuric stage), 169 had microalbuminuria of 30–299 mg/g-Cr (Stage II: microalbuminuric stage), 84 had macroalbuminuria >300 mg/g-Cr and serum Cr <1.1 mg/dl (Stage IIIA: macroalbuminuric stage without renal dysfunction), 97 had macroalbuminuria >300 mg/g-Cr and serum Cr >1.2 mg/dl (Stage IIIB: macroalbuminuric stage with renal dysfunction), and 84 had renal failure (Stage IV). Characteristics of these patients are shown in Table 1.

Measurement of Urinary Type IV Collagen

(a) Preparation of Fab'-peroxidase conjugate

Purified mouse monoclonal antibody against the triple helix (TH) domain of human placental type IV collagen (Fuji Chemical Industries, Co. Ltd., Takaoka, Toyama, Japan) was digested with pepsin from porcine mucosa to obtain F(ab')₂. F(ab')₂ was reduced with 2-aminoethanethiol, and the Fab'-peroxidase conjugate was prepared by the method of Hashida

et al. (11) using *N*-(ε-maleimidocaproyloxy)succinimide (EMCS) as a maleimide compound for the conjugation of Fab' to horseradish peroxidase (HRP) through thiol groups in the hinge region.

(b) 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. (12) at 4°C for 24 hr with 0.1 g/l purified mouse monoclonal antibody against the TH domain of human placental type IV collagen (Fuji Chemical Industries, Co. Ltd.) in 0.1 M Na-phosphate buffer (pH 7.5) containing 0.1% NaN₃. The balls were washed five times with 10 mM Na-phosphate buffer (pH 7.0) containing 0.1% bovine serum albumin (BSA) and 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.

(c) One-step sandwich enzyme immunoassay (EIA) technique

A 100-μl aliquot of standard pepsin-solubilized type IV collagen or urine was mixed with 300 μl of anti-TH domain of type IV collagen mouse monoclonal antibody, and Fab'-HR conjugate (0.8 ng/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 anti-7S domain of type IV collagen mouse monoclonal antibody was then added to the solution. Each of the plastic test tubes was left for 24 hr at 8°C for immunoreaction. Thereafter, the polystyrene ball was washed two times with 3.5 ml of 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 3,3',5,5'-tetramethylbenzidine (TMBZ) (0.134 g/l) and 100 μl of hydrogen peroxide (0.15 g/l) for 1 hr at 25°C by the method of Bos et al. (13). The reaction was stopped by adding 1 ml of sulfuric acid (0.67 M), and the absorbance at 450 nm was measured by a Micro-Flow spectrophotometer (Shimadzu, Model UV730, Kyoto, Japan) (14). The normal

TABLE 1. Characteristics of diabetic patients in Asian countries^a

	Kuala Lumpur (n=37)	Hong Kong (n=111)	Kaohsiung (n=53)	Shenyang (n=84)	Seoul (n=134)	Tokyo (n=279)	Total (n=698)
Male/female	14/23	61/50	24/29	39/45	80/54	181/98	399/299
Age (years)	55.4 ± 1.5	64.5 ± 11.7	58.5 ± 16.7	52.8 ± 15.8	57.9 ± 13.9	55.6 ± 10.9	57.4 ± 13.2
Duration of diseases (years)	11.2 ± 1.7	9.2 ± 6.8	8.5 ± 6.8	7.3 ± 6.2	9.4 ± 7.4	10.0 ± 7.0	9.4 ± 7.2
BMI ^b	26.2 ± 0.6	24.8 ± 5.1	22.7 ± 3.5	24.1 ± 3.9	23.4 ± 4.1	23.7 ± 3.6	23.9 ± 4.0
FPG (mg/dl)	153.4 ± 9.6	166.3 ± 61.2	244.4 ± 186.1	212.3 ± 74.8	157.4 ± 66.6	170.8 ± 66.3	177.3 ± 85.2
SBP (mmHg)	158.9 ± 4.8	137.1 ± 18.2	140.8 ± 16.5	138.0 ± 27.1	131.9 ± 15.9	134.1 ± 17.2	136.5 ± 20.2
DBP (mmHg)	85.2 ± 2.4	74.5 ± 10.9	83.4 ± 11.1	81.6 ± 13.5	82.3 ± 10.2	78.7 ± 10.1	79.7 ± 11.5

^aValues presented as mean ± SD.

^bAbbreviations: BMI, body mass index; FPG, fasting plasma glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure.

cutoff range of urinary type IV collagen of healthy adults was less than 3.5 $\mu\text{g/g}\cdot\text{Cr}$.

Clinical Parameters

Serum creatinine (s-Cr), blood pressure, fasting plasma glucose (FPG), glycohemoglobin A1c (HbA1c), and body mass index (BMI) were examined by routine methods in each clinic. Statistical analyses were performed by Pearson's correlation coefficient and the two-sample *t* independent-group test.

RESULTS

Urinary Type IV Collagen and Albumin

The level of urinary type IV collagen in Stage I ($5.00 \pm 0.23 \mu\text{g/g}\cdot\text{Cr}$; mean $\pm$ SE) was higher than that in normal adults ($3.44 \pm 0.11 \mu\text{g/g}\cdot\text{Cr}$; mean $\pm$ SE). The level of urinary type IV collagen in Stage II was significantly higher than that in Stage I ($13.58 \pm 1.78 \mu\text{g/g}\cdot\text{Cr}$ versus $5.00 \pm 0.23 \mu\text{g/g}\cdot\text{Cr}$, $P < 0.0001$). The levels of urinary type IV collagen in Stages II, IIIA, IIIB, and IV were significantly higher than those in Stage I or healthy adults ($P < 0.0001$). These levels increased gradually due to progression of the clinical stage of diabetic nephropathy (Table 2). The level of urinary type IV collagen in Stage IIIB was lower than that in Stage IIIA, but the difference was not statistically significant. There was a significant correlation between the concentration of urinary type IV collagen and that of urinary albumin in all stages of diabetic nephropathy ($R = 0.104$, $P < 0.01$).

Percentages of the patients classified according to the measurements of urinary albumin and urinary type IV collagen are shown in Figure 1 (normal cutoff range: $<3.5 \mu\text{g/g}\cdot\text{Cr}$). In Stage I, 160 out of 264 patients (60.6%) and in Stage II, 142 out of 169 patients (84.0%) showed elevated urinary type IV collagen. In Stages IIIA and IIIB, 81 out of 84 patients (96.4%) and 83 out of 97 patients (85.6%), respectively, showed elevated urinary type IV collagen. Elevated urinary type IV collagen was seen in 82 out of 84 patients (97.6%) in Stage IV.

The levels of urinary type IV collagen in each city are shown in Table 3. Urinary type IV collagen levels in all stages in patients in the trials in Kaohsiung and Shenyang were

higher than those in other cities. In Kaohsiung and Shenyang, high percentages of patients showed elevated urinary type IV collagen (normal range: $<3.5 \mu\text{g/g}\cdot\text{Cr}$) in Stages II, IIIA, and IIIB.

Relationship Between Urinary Type IV Collagen Levels and Other Clinical Parameters

There was a significant correlation between the levels of urinary type IV collagen and those of s-Cr, systolic blood pressure, diastolic blood pressure, and age ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$, and $P < 0.025$, respectively). However, there was no significant correlation between the levels of urinary type IV collagen and those of fasting plasma glucose (FPG), HbA1c, or the body mass index (BMI).

DISCUSSION

Type IV collagen is the major collagenous component of the extracellular matrix (ECM). It is generally considered that the accumulation of ECM including type IV and VI collagens, fibronectin and laminin, leads to glomerular sclerosis in various renal diseases (15,16). Excess accumulation of ECM may be due to an increase of production and/or decrease of degradation of the ECM. An increase in the intensity of immunohistochemical staining of type IV collagen was observed in renal tissues from patients with diabetic nephropathy. The increase of urinary type IV collagen concentration in diabetic patients might be reflected by the increase of its production and/or degradation. Recently, we reported that the concentration of urinary type IV collagen in diabetic patients with microalbuminuria was significantly higher than that in patients with normoalbuminuria using a highly sensitive one-step EIA (6–8). This method is characterized by the application of two kinds of monoclonal antibodies against the 7S and TH domains of type IV collagen and measurements without any pretreatment. Gel filtration analysis of urinary samples from both normal controls and diabetic patients showed similar patterns with a single peak at a molecular mass of about 540 kDa, which is close to that of intact type IV collagen (data not shown). It is speculated that type IV collagen detected in the urine is released from the renal tissues but not from blood samples.

Levels of urinary type IV collagen in patients in Stage II (microalbuminuric stage) were significantly higher than those in Stage I (normoalbuminuric stage) in this study. The levels of urinary type IV collagen in Stage I were also higher than those in normal adults. These findings were consistent with our previous data (6–8). The levels of urinary type IV collagen increased due to progression of the clinical stage of diabetic nephropathy. There was a significant correlation between concentration of urinary albumin and that of urinary type IV collagen. However, a substantial number of patients (60.4% and 84.0%) in Stage I (normoalbuminuric stage) or II (microalbuminuric stage) showed a high concentration of uri-

TABLE 2. Levels of urinary type IV collagen in healthy adults and diabetic patients^a

Healthy adults	(<i>n</i> = 191)	$3.44 \pm 0.11 (\mu\text{g/g} \cdot \text{Cr})$
Diabetic patients		
Stage I	(<i>n</i> = 264)	5.00 ± 0.23
Stage II	(<i>n</i> = 169)	$13.58 \pm 1.78^*$
Stage IIIA	(<i>n</i> = 84)	$21.70 \pm 2.19^*$
Stage IIIB	(<i>n</i> = 97)	$20.47 \pm 2.79^*$
Stage IV	(<i>n</i> = 84)	$42.94 \pm 4.07^*$

^aData are mean $\pm$ standard error of the mean (SE).

* $P < 0.0001$ versus Stage I or healthy adults.

Fig. 1. Percentages of patients showing a high concentration of urinary type IV collagen in Stages I-IV.

TABLE 3. Levels of urinary type IV collagen (a) and albumin (b) of diabetic patients in Asian countries^{a,b}

Stage of diabetes		Kuala Lumpur (n=37)	Hong Kong (n=111)	Kaohsiung (n=53)	Shenyang (n=84)	Seoul (n=134)	Tokyo (n=279)
Stage I	(a)	4.37 ± 0.79	5.60 ± 0.76	8.55 ± 3.41	4.51 ± 0.55	6.41 ± 0.50	4.07 ± 0.27
	(b)	8.34 ± 1.53	16.62 ± 1.67	12.03 ± 2.68	11.91 ± 1.86	9.52 ± 0.83	9.52 ± 0.61
Stage II	(a)	8.39 ± 0.85	8.86 ± 1.42	51.04 ± 19.05	19.90 ± 3.32	19.94 ± 8.12	6.59 ± 0.54
	(b)	99.90 ± 46.67	78.99 ± 10.42	123.33 ± 23.20	94.76 ± 10.16	99.31 ± 15.14	106.99 ± 8.33
Stage IIIA	(a)	18.02 ± 4.59	16.35 ± 5.70	35.10 ± 14.52	44.05 ± 3.26	8.92 ± 2.26	9.79 ± 0.94
	(b)	1,674.90 ± 630.51	2,005.76 ± 668.06	3,630.90 ± 1,890.94	379.51 ± 29.14	1,346.60 ± 564.42	1,339.96 ± 209.04
Stage IIIB	(a)	25.97 ± 8.23	10.56 ± 1.77	51.59 ± 18.17	43.04 ± 9.90	14.08 ± 2.79	15.11 ± 3.02
	(b)	2,471.83 ± 1,057.54	627.35 ± 256.10	1,293.71 ± 554.50	338.37 ± 80.49	1,076.45 ± 398.70	2,413.56 ± 667.70
Stage IV	(a)	30.74 ± 5.91	26.97 ± 3.79	56.81 ± 10.62	71.82 ± 21.84	61.19 ± 10.84	24.75 ± 5.14
	(b)	3,290.16 ± 594.37	1,938.87 ± 378.66	2,991.62 ± 445.50	515.96 ± 52.79	5,369.51 ± 1,553.26	2,311.81 ± 324.71

^aData are mean ± standard error of the mean (SE).

^bKey: (a) µg/g · Cr; (b) µg/g · Cr.

nary type IV collagen, clearly more than the normal range. Conversely, 16.0% of the microalbuminuric patients did not exhibit any increase of urinary type IV collagen excretion. This dissociation seems to be quite interesting and should not be ignored. It is postulated that urinary type IV collagen might be increased prior to an increase of urinary albumin in diabetic patients. Thus the measurement of urinary type IV collagen should be useful for detection of early stage of diabetic nephropathy.

Interestingly, the urinary type IV collagen levels detected in the same institute in Japan in all stages in patients in the trials in Kaohsiung and Shenyang were higher than those in other cities including Hong Kong. There was no significant correlation between the levels of urinary type IV collagen and those of fasting plasma glucose (FPG) in this study. However, the levels of FPG were markedly increased in Kaohsiung and Shenyang. It is postulated that the increase of FPG might influence the metabolism of type IV collagen in renal tissues and its excretion of into the urine. In this study, there was a significant correlation between the levels of urinary type IV collagen and those of serum creatinine (s-Cr), blood pressure, and age. Thus it is necessary to determine the relationship between genetic and/or environmental factors and the production/degradation of type IV collagen in the kidneys in Asian countries.

It appears that the measurement of urinary type IV collagen concentration can be a useful marker for detecting renal injuries in diabetic patients, especially in the early stage, according to our Asian multicenter trials.

REFERENCES

- Incidence by primary disease in 1999. In: An overview of regular dialysis treatment in Japan. *Jpn Soc Dial Ther* 1999;817 (in Japanese).
- Mauer SM, Steffes MW, Ellis EN, Sutherland DER, Brown DM, Goetz FC. Structural-functional relationships in diabetic nephropathy. *J Clin Invest* 1984;74:1143-1155.
- Hogemann B, Balleisen L, Rauterberg J, Voss B, Gerlach U. Basement membrane components (7S collagen, laminin P1) are increased in sera of diabetic and activate platelets in vitro. *Haemostasis* 1986; 16:428-432.
- Hayashi Y, Makino H, Ota Z. Serum and urinary concentrations of type IV collagen and laminin as a marker of microangiopathy in diabetes. *Diabet Med* 1992;9:366-370.
- Yagame M, Kim Y, Zhu D, Suzuki D, Eguchi K, Nomoto Y, Sakai H, Groppoli T, Steffes MW, Mauer SM. Differential distribution of type IV collagen chains in patients with diabetic nephropathy in non-insulin-dependent diabetes mellitus. *Nephron* 1995;70:42-48.
- Yagame M, Suzuki D, Jinde K, Saotome N, Sato H, Noguchi M, Sakai H, Kuramoto T, Sekizuka K, Iijima T, Suzuki S, Tomino Y. Significance of urinary type IV collagen in patients with diabetic nephropathy using a highly sensitive one-step sandwich enzyme immunoassay. *J Clin Lab Anal* 1997;11:110-116.
- Iijima T, Suzuki S, Sekizuka K, Hishiki T, Yagame M, Jinde K, Saotome N, Suzuki D, Sakai H, Tomino Y. Follow-up study on type IV collagen in patients with early stage diabetic nephropathy. *J Clin Lab Anal* 1998;12:378-382.
- Takizawa H, Satoh T, Kurusu A, Hishiki T, Shike T, Gohda T, Maeda A, Makita Y, Suzuki S, Fukui M, Tomino Y. Increase of urinary type IV collagen in normoalbuminuric patients with impaired glucose tolerance (IGT). *Nephron* 1998;79:474-475.
- Kuzuya N. Report of the committee of diagnostic criteria of diabetes mellitus by the glucose tolerance test. *J Jpn Diab Soc* 1970;13:17-20.
- Stage classification of diabetic nephropathy. In: Reports of Ministry of Health and Welfare Japan. Tokyo, Japan: Ministry of Health and Welfare; 1991. p 251-256 (in Japanese).
- Hashida S, Imagawa M, Inoue S, Ruan K-H, Ishikawa E. More useful maleimide compounds for the conjugation of Fab' to horseradish peroxidase through thiol groups in the hinge. *J Appl Biochem* 1984;6:56-63.
- Ishikawa E, Imagawa M, Hashida S, Hamaguchi Y, Ueno T. Enzyme-labeling of antibodies and their fragments for enzyme immunoassay and immunohistochemical staining. *J Immunoassay* 1983;4:209-327.
- Bos ES, Van der Doelen AA, Rooy NV, Schuurs AHWM. 3,3',5,5'-Tetramethylbenzidine as an Ames test negative chromogen for horseradish peroxidase in enzyme-immunoassay. *J Immunoassay* 1981;2: 187-204.
- Obata K, Iwata K, Ichida T, Inoue K, Matsumoto E, Muragaki Y, Ooshima A. One step sandwich enzyme immunoassay for human type IV collagen using monoclonal antibodies. *Clin Chim Acta* 1989;181: 293-304.
- Nelich A, Schleicher E. Immunohistochemical localization of extracellular matrix components in human diabetic glomerular lesions. *Am J Pathol* 1991;139:889-899.
- Falk RJ, Scheinman JJ, Mauer SM, Michael AF. Polyantigenic expansion of basement membrane constituents in diabetic nephropathy. *Diabetes* 1983;32(Suppl 2):34-39.