Relationship Between Levels of Urinary Type IV Collagen and Renal Injuries in Patients With IgA Nephropathy

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Because type IV collagen is synthesized by podocytes and mesangial cells, we investigated the relationship between levels of urinary type IV collagen (uIV) and renal injuries in patients with IgA nephropathy. uIV was measured by a highly sensitive one-step sandwich enzyme immunoassay prior to renal biopsy. Patients with IgA nephropathy were classified into four grades (grade 1 = good prognosis, grade 2 = relatively good prognosis, grade 3 = relatively poor prognosis, and grade 4 = poor prognosis) by the prognostic criteria of the Ministry of Health, Labor, and Welfare of Japan. Levels of uIV in grade 4 were significantly higher than those in grades

1–3. These levels tended to increase gradually due to progression of renal injuries. The grades were further divided into two groups: group I (good or relatively good prognoses) and group II (relatively poor or poor prognoses). Patients with proteinuria of <1.0 g/day were defined as groups Ip and IIp. The levels of uIV in group II were significantly higher than those in group I, and those in group IIp were significantly higher than those in group Ip. It appears that the level of uIV can be a useful marker for detection of renal injuries in IgA nephropathy. *J. Clin. Lab. Anal.* 18:14–18, 2004. © 2004 Wiley-Liss, Inc.

Key words: urinary type IV collagen; proteinuria; renal biopsy; renal injury; IgA nephropathy

INTRODUCTION

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 (1–4). It is generally considered that the accumulation of extracellular matrix (ECM) may be regulated through a balance of its production and degradation by matrix proteinases. Therefore, increased urinary type IV collagen (uIV) excretion indicates ongoing alterations in the turnover of glomerular and tubular type IV collagen. Several previous studies indicated that the urinary excretion of type IV collagen was increased in patients with various types of glomerulonephritis compared to normal controls (5). Takizawa et al. (6) also reported an increase of uIV in normoalbuminuric patients with impaired glucose tolerance. Mesangial cell proliferation and matrix accumulation, including type IV collagen, have been observed in progressive IgA nephropathy, and increased deposition of collagens have been closely related to the sclerotic activity of the disease process in IgA nephropathy (7). In the present study we sought to determine

whether uIV is a useful marker in IgA nephropathy, by measuring urinary levels of type IV collagen and comparing them with renal histological findings.

MATERIALS AND METHODS

Patients and Urinary Samples

Urinary samples were obtained from 34 IgA nephropathy patients who were admitted to Juntendo University Hospital for renal biopsy. None of the patients had a history of diabetes mellitus, liver disease, or systemic disorders such as rheumatoid arthritis or systemic lupus erythematosus (SLE). Spot urine samples of the first urination in the morning were collected and then corrected by the ratio of urinary creatinine (Cr)

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concentration to avoid the influence of urinary volume. Proteinuria was measured by collecting urinary samples for 1 day (24 hr).

IgA nephropathy was diagnosed and classified according to the *Clinical Guidelines of IgA Nephropathy* in Japan (8). Grade 1 is the good prognosis group, characterized by slight mesangial cell proliferation and increased matrix. No glomerulosclerosis, crescent formation, or adhesion to Bowman's capsule is observed. Grade 2, the relatively good prognosis group, shows slight mesangial cell proliferation and increased matrix. Glomerulosclerosis, crescent formation, or adhesion to Bowman's capsule is seen in <10% of all biopsied glomeruli. Grade 3, the relatively poor prognosis group, has moderate, diffuse mesangial cell proliferation and increased matrix. Glomerulosclerosis, crescent formation, or adhesion to Bowman's capsule is seen in 10–30% of all biopsied glomeruli. Grade 4, the poor prognosis group, is characterized by severe, diffuse mesangial cell proliferation and increased matrix. Glomerulosclerosis, crescent formation, or adhesion to Bowman's capsule is seen in >30% of all biopsied glomeruli. In this study, six patients were in grade 1, eight in grade 2, 12 in grade 3, and eight in grade 4. These grades were further divided into two groups: group I ($n = 14$), consisting of grades 1 and 2; and group II ($n = 20$) consisting of grades 3 and 4. Group I patients who had urinary protein of <1.0 g/day were defined as group Ip ($n = 14$), and group II patients with urinary protein of <1.0 g/day were classified as group Iip ($n = 11$).

Measurement of uIV

Preparation of Fab'-peroxidase conjugate

Purified mouse monoclonal antibody against the triple helix (TH) domain of human placental type IV collagen (Fuji Chemical Industries Ltd., Takaoka, Toyama, Japan) was digested with pepsin from porcine mucosa to obtain $F(ab')_2$, which was reduced with 2-aminoethanethiol. The Fab'-peroxidase conjugate was prepared by the method of Hashida et al. (9) using N-(ϵ -maleimidocaproyloxy) succinimide (EMCS) as a maleimide compound for conjugation of Fab' to horseradish peroxidase (HRP) via thiol groups in the hinge region.

Preparation of monoclonal antibody-coated polystyrene balls

Polystyrene balls were coated by physical absorption. The procedure was performed according to the method of Ishikawa et al. (10) 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, Ltd.) in 0.1 M Na-phosphate buffer (pH 7.5) containing 0.1% bovine serum albumin (BSA) and 0.1 M NaCl. The balls were stored at 4°C and washed with 10 mM Na-phosphate buffer (pH 7.0) containing 0.1% BSA and 0.1 M NaCl immediately prior to use.

One-step sandwich enzyme immunoassay (EIA) technique

A 100- μ L aliquot of standard pepsin-solubilized type IV collagen or urine was mixed with 300 μ L 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 the anti-7S domain of type IV collagen mouse monoclonal antibody was then added to the solution. The plastic test tubes were 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 transferred to another plastic test tube, which was incubated with 300 μ L of 3,3',5,5'-tetramethylbenzidine (TMBZ) (0.134 g/l) and 100 μ L of hydrogen peroxide (0.15 g/l) for 1 hr at 25°C by the method of Bos et al. (11). 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 (model UV730; Shimadzu, Kyoto, Japan) (12).

Statistical Analyses

The data were presented as mean $\pm$ SD. Analysis of variance (ANOVA) and the unpaired t -test were used to test statistical significance. The level of significance was set at $P < 0.05$.

RESULTS

Clinical Parameters

The characteristics of the patients in this study prior to renal biopsy are shown in Tables 1A–C. As shown in Table 1A, the grade 2 patients were significantly younger than the grade 4 patients ($P < 0.05$). Serum creatinine (S-Cr) levels were significantly higher in grade 4 than in grades 1–3 ($P < 0.05$). Systolic blood pressure (SBP) was significantly lower in grade 2 than in grades 3 and 4 ($P < 0.05$). Diastolic blood pressure (DBP) was significantly lower in grade 2 than in grade 3 ($P < 0.05$). The levels of creatinine clearance (Ccr) were significantly lower in grade 4 than in grades 1–3 ($P < 0.05$). As shown in Table 1B, the ages, S-Cr, SBP, and DBP were

TABLE 1A. Characteristics of patients prior to renal biopsy

	Grade 1 (n=6)	Grade 2 (n=8)	Grade 3 (n=12)	Grade 4 (n=8)
Male/female	3/3	1/7	8/4	4/4
Age	26.3±9.4	25.6±7.4 ^a	35.9±13.6	38.0±13.9
S-Cr (mg/dl)	0.66±0.10	0.63±0.19	0.77±0.15	1.10±0.51 ^b
SBP (mmHg)	113.2±9.7	102.5±8.5 ^c	120.7±15.7	127.3±25.1
DBP (mmHg)	64.3±3.9	61.3±5.0 ^d	71.3±10.9	68.5±9.7
Ccr (ml/min)	99.2±25.5	83.9±13.6	87.7±19.6	61.6±24.8 ^e

^a*P*<0.05 vs. grade 4.^b*P*<0.05 vs. grade 1, 2, and 3.^c*P*<0.05 vs. grade 3 and 4.^d*P*<0.05 vs. grade 3.^e*P*<0.05 vs. grade 1, 2, and 3.**TABLE 1B. Characteristics of patients in groups I and II**

	Group I (grade 1 + 2) (n=14)	Group II (grade 3 + 4) (n=20)
Male/female	4/10	12/8
Age	25.9±8.0 ^a	36.8±13.4
S-Cr (mg/dl)	0.64±0.16 ^a	0.90±0.37
SBP (mmHg)	108.1±10.6 ^a	123.3±19.6
DBP (mmHg)	62.6±4.7 ^a	70.2±10.2
Ccr (ml/min)	90.5±20.3	77.3±24.9

^a*P*<0.05 vs. group II.**TABLE 1C. Characteristics of patients with proteinuria of less than 1.0 g/day**

	Group I P (n=14)	Group II P (n=11)
Male/female	4/10	8/3
Age (years old)	25.9±8.0	34.9±14.5
S-Cr (mg/dl)	0.64±0.16 ^a	0.81±0.17
SBP (mmHg)	108.1±10.6 ^a	118.7±15.9
DBP (mmHg)	62.6±4.7	69.5±11.5
Ccr (ml/min)	90.5±20.3	82.2±25.9

^a*P*<0.05 vs. group II p.

S-Cr, serum creatinine; Ccr, creatinine clearance; SBP, systolic blood pressure; DBP, diastolic blood pressure.

significantly lower in group I than in group II (*P*<0.05). Table 1C shows the characteristics of the patients who had proteinuria of <1.0 g/day (groups Ip and IIp). S-Cr and SBP levels were significantly lower in group Ip than in group IIp (*P*<0.05).

uIV in Grades 1–4

The level of uIV was significantly higher in grade 4 ($4.30 \pm 2.64 \mu\text{g/g} \cdot \text{Cr}$) than in grades 1–3 (1.77 ± 0.446 , 2.25 ± 1.09 , and $2.77 \pm 1.27 \mu\text{g/g} \cdot \text{Cr}$, respectively,

Fig. 1. uIV concentration in grades 1–4. **Fig. 2.** uIV concentration in groups I and II.

P<0.05) (Fig. 1). These levels tended to increase gradually due to progression of renal injury according to pathological grading in patients with IgA nephropathy.

uIV in Groups I (Grades 1 and 2) and II (Grades 3 and 4)

The level uIV was significantly higher in group II than in group I ($3.38 \pm 2.02 \mu\text{g/g} \cdot \text{Cr}$ vs. $2.04 \pm 0.879 \mu\text{g/g} \cdot \text{Cr}$; *P*<0.05) (Fig. 2). In group I, all 14 patients were classified as group Ip (urinary protein of <1.0 g/day). Group IIp findings were observed in 11 of 20 patients (55%). The level of uIV in group IIp was

Fig. 3. uIV concentration in patients with proteinuria of <1.0 g/day.

significantly higher than that in group Ip ($2.98 \pm 1.23 \mu\text{g/g} \cdot \text{Cr}$ vs. $2.04 \pm 0.879 \mu\text{g/g} \cdot \text{Cr}$; $P < 0.05$) (Fig. 3).

DISCUSSION

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 (13, 14). An increase in the intensity of immunohistochemical staining of type IV collagen was observed in renal tissues from patients with IgA nephropathy (7, 15). This increase of urinary excretion of type IV collagen in IgA nephropathy may reflect increases in its production and/or degradation. We recently found, using a highly sensitive one-step EIA, that the concentration of uIV in diabetic patients with microalbuminuria was significantly higher than in patients with normoalbuminuria (16). This method involves 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 patients with IgA nephropathy showed a single peak, demonstrating the presence of a high-molecular-weight antigen in urine, suggesting measurement of intact type IV collagen (data not shown).

Previously, Makino et al. (5) reported that the urinary concentration of type IV collagen was increased in patients with various types of glomerulonephritis, compared to normal controls. In particular, patients with membranous nephropathy (MN) and chronic renal failure (CRF) demonstrated a significant increase of uIV

excretion compared to normal controls. This uIV appeared to be derived from injured cells and/or mesangial matrix (5). In those patients, morphological changes, including podocyte and mesangial cell injuries and increased mesangial matrices, were generally marked. In the present study, the levels of uIV were significantly higher in grade 4 (poor prognosis) than in grades 1 (good prognosis), 2 (relatively good prognosis), and 3 (relatively poor prognosis). In particular, the levels of uIV were significantly higher in group IIp (urinary protein of <1.0 g/day) than in group Ip (urinary protein of <1.0 g/day). For patients with urinary protein of <1.0 g/day, the levels of uIV were significantly higher in group II (relatively poor and poor prognoses) than in group I (good and relatively good prognoses). The patients in grades 3 and 4 had increased matrix, glomerulosclerosis, and crescent formation compared to patients in grades 1 and 2. It is speculated that increases in the urinary concentration of type IV collagen may reflect alterations of ECM metabolism occurring in progressive IgA nephropathy. It appears that the measurement of uIV, which has the distinct advantage of being noninvasive, may be a useful tool for detection of renal injuries in patients with IgA nephropathy.

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