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Follow-Up Study on Urinary Type IV Collagen in Patients With Early Stage Diabetic Nephropathy

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Type IV collagen is a major component released from the glomerular and tubular basement membranes. To investigate the alteration of renal type IV collagen turnover in early stage diabetic nephropathy, urinary type IV collagen was measured by a highly sensitive one-step sandwich enzyme immunoassay (EIA). Urinary samples were obtained from 94 diabetic patients without overt proteinuria. Among those patients, 61 were normoalbuminuric and 33 patients were in the microalbuminuric group. Levels of urinary type IV collagen were serially examined at the start of this study and again one year later. The levels of urinary type IV collagen in patients in the microalbuminuric group were significantly higher than those in the normoalbuminuric group ($P < 0.01$). There was a significant correlation between the concentration of urinary albumin and urinary type IV collagen in both groups ($P < 0.05$). Twenty-eight patients (45.3%) in the normoalbuminuric group who showed an abnormal elevation of urinary type IV collagen in comparison to the reference range of normal healthy adults (normal range; less than 3.5 μg/g · Cr). Seven (25%) out of these 28 normoalbuminuric patients with increased urinary type IV collagen progressed to the microalbuminuric group one year later. The levels of urinary type IV collagen in such patients were significantly increased. In the 21 patients who stayed within the normoalbuminuric group, the urinary type IV collagen levels were significantly decreased one year later. It appears that the levels of urinary type IV collagen might reflect ongoing alteration of the extracellular matrix (ECM) turnover and might define more specifically the early stage diabetic nephropathy than the detection of microalbuminuria. It is concluded that the serial measurement of urinary type IV collagen can be a useful marker for detecting renal injury in diabetes. J. Clin. Lab. Anal. 12:378–382, 1998.

Key words: urinary type IV collagen; diabetic nephropathy

INTRODUCTION

Diabetic nephropathy is one of the major long-term microvascular complications occurring in nearly 40% of diabetic patients (1). Microalbuminuria is the only clinical sign of their later 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.

Morphologically, the most prominent feature of diabetic nephropathy is excess accumulation of extracellular matrix (ECM) in the glomerular mesangial areas and basement membranes (2,3). It is generally considered that the accumulation of ECM might be regulated by the balance of its production and degradation by matrix proteinases. Since type IV collagen is the principal component of glomerular basement membrane and mesangial matrix, the levels of type IV collagen in sera and urinary samples may reflect the rate of matrix turnover in diseased kidneys (4,5,6). 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). The purpose of the present study was to determine whether the serial measurement of urinary type IV collagen may act as a

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sensitive marker reflecting alteration of the ECM turnover in early stage diabetic nephropathy.

MATERIALS AND METHODS

Patients

Ninety-four outpatients with non-insulin-dependent diabetes mellitus (NIDDM) without overt proteinuria (62 males and 32 females), aged 32 to 81 years (average: 55.6 ± 10.7 years, mean ± SD) were used in this study. Diagnosis of NIDDM was made according to the criteria of the Japanese Diabetic Research Council (7). 296 age-matched healthy adults (202 males, 94 females) were also used as controls. None of the patients had any history of liver diseases or systemic disorders such as rheumatoid arthritis or systemic lupus erythematosus (SLE). All patients were screened as negative for overt proteinuria by N-Multistix SG-L (Bayer-Sankyo, Co. Ltd., Tokyo, Japan) and followed by a quantitative measurement of urinary albumin concentration by turbidimetric immunoassay (TIA). 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 influence of the urinary volume. Urine was sampled twice from all 94 patients at the start of this study and again one year later. At the beginning of the study, 61 patients had urinary albumin concentration of less than 29 mg/g · Cr (normoalbuminuric group) and 33 patients had microalbuminuria from 30 to 299 mg/g · Cr (microalbuminuric group). Mean levels of urinary albumin in the normoalbuminuric and microalbuminuric groups were 7.1 ± 5.5 mg/g · Cr and 61.2 mg/g · Cr, respectively. No difference in clinical features including age, gender, blood pressure, and glycemic control was observed between the normo- and microalbuminuric groups.

Measurement of Urinary Type IV Collagen

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. (8) 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. (9) at 4°C for 24 hours with 0.1 g/l purified mouse monoclonal antibody against the triple helix (TH) domain of human placental type IV collagen (Fuji Chemical Industries, Co. Ltd., Takaoka, Toyama, Japan) in 0.1 M Na-phosphate buffer (pH 7.5) containing 0.1% NaN_3. The balls were washed five times with 10 mM Na-phosphate buffer (pH 7.0) containing 0.1% 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.

One step sandwich EIA technique

A 100 µl aliquot of standard pepsin-solubilyzed type IV collagen or urine was mixed with 300 µl of anti-TH domain mouse monoclonal antibody, 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 mouse monoclonal antibody was then added to the solution. Each of the plastic test tubes was left for 24 hours at 8°C for immunoreaction. Thereafter, the polystyrene ball was washed two times 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 one hour at 25°C by the method of Bos et al. (10). The reaction was stopped by adding 1 ml sulfuric acid (0.67 M) and the absorbance at 450 nm was measured by a Micro-Flow spectrophotometer (Shimadzu, Model UV730, Kyoto, Japan)(11). Normal range of urinary type IV collagen of healthy adults was less than 3.5 µg/g · Cr.

RESULTS

Measurement of Urinary Albumin and Type IV Collagen

Urinary type IV collagen concentration in the microalbuminuric group was significantly higher than that in the normoalbuminuric group (6.3 ± 4.58 µg/g · Cr vs. 3.5 ± 2.31 µg/g · Cr, $P < 0.01$). There was a significant correlation between the concentration of urinary type IV collagen and that of urinary albumin in both groups ($R = 0.104$, $P < 0.01$) (Fig. 1). Percentages of the patients classified according to the measurements of urinary albumin and urinary type IV collagen are shown in Fig. 2. In the normoalbuminuric group, 28 patients (46.7 %) showed an elevated urinary type IV collagen (normal range; less than 3.5 µg/g · Cr). No increase of urinary type IV collagen levels was observed in 11 patients (33.3%) in the microalbuminuric group. There was no significant correlation among the levels of urinary albumin or urinary type IV collagen, HbA1c and mean blood pressure in both groups.

Follow-Up Study of Urinary Albumin and Type IV Collagen

Urinary samples from 28 normoalbuminuric patients with increased urinary type IV collagen levels were re-examined

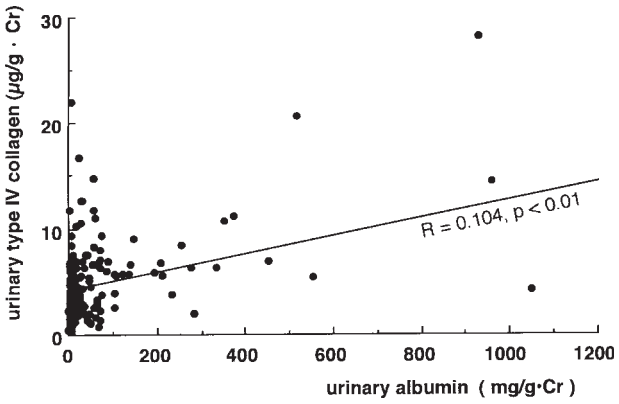

Fig. 1. Relationship between the levels of urinary type IV collagen in normoalbuminuric and microalbuminuric groups.

one year later for detecting the outcome of the urinary albumin excretion. Seven of the 28 patients (25%) changed from normo- to microalbuminuria. The urinary type IV collagen concentration significantly increased from 5.74 ± 2.85 μg/g · Cr to 7.79 ± 3.81 μg/g Cr ($P < 0.05$) one year later in microalbuminuric patients (Fig. 3). However, in two cases, the urinary type IV collagen concentration was slightly decreased. In contrast, the other 21 patients stayed within the normoalbuminuric group and showed a significant decrease in the concentration of urinary type IV collagen from 5.31 ± 1.80 μg/g · Cr to 3.87 ±2.33 μg/g · Cr ($P < 0.05$, Fig. 4).

DISCUSSION

Type IV collagen is the major collagenous component of the extracellular matirx (ECM). An increase in the intensity of immunohistochemical staining for type IV collagen was observed in renal tissues from patients with diabetic nephropathy. It was generally considered that the accumulation of ECM including type IV and VI collagens, fibronectin and laminin, leads to glomerular sclerosis in various renal diseases (12,13,14). Excess accumulation of ECM could be due to increase of production and/or decrease of degradation of the ECM. Jinde et al. (15) 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 in situ hybridization of renal tissues from patients with diabetic nephropathy. Therefore, 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 those with normoalbuminuria using highly sensitive one-step EIA (6). This method is characterized by the use of two kinds of monoclonal antibodies against the 7S and TH domain of type IV collagen and measurements without any treatment before use (11).

The levels of urinary type IV collagen in patients in the microalbuminuric group were significantly higher than those in the normoalbuminuric group. These findings were consitent with our previous data (6). Gel filtration analysis of urinary samples from both normal controls and diabetic patients showed similar patterns with a single peak at a molecular size of about 540 kD which is close to intact type IV collagen (data not shown). It is postulated that type IV collagen detected in the urine is released from the renal tissues, but not from blood samples. There was a significant correlation be-

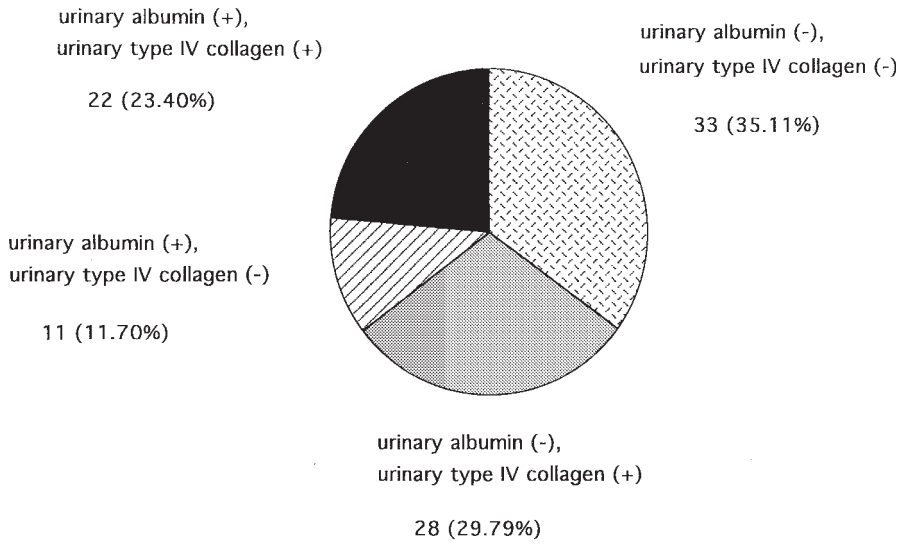

Fig. 2. Percentages of the patients classified according to the measurements of urinary albumin and urinary type IV collagen.

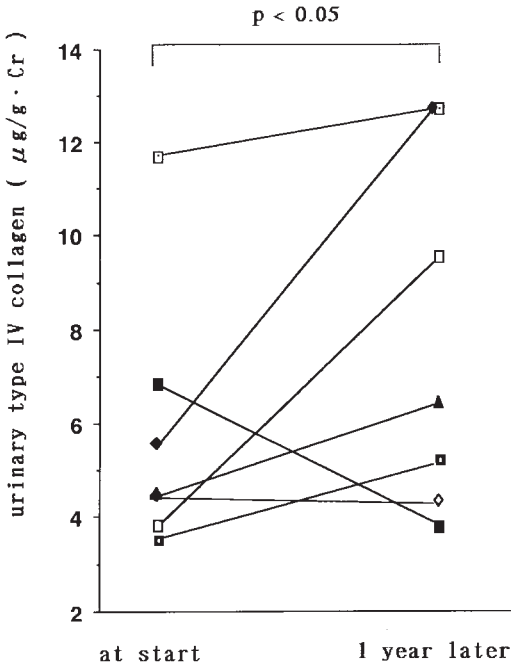

Fig. 3. Alteration of urinary type IV collagen levels in patients changed from normo- to microalbuminuria at the start of this study and one year later.

tween the concentration of urinary albumin and urinary type IV collagen in this study. However, a substantial number of patients (45.3%) in the normoalbuminuric group showed a high concentration of urinary type IV collagen clearly more than the normal range. On the contrary, 33% 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. A follow-up examination one year later was performed to determine the meaning of increased urinary type IV collagen excretion in normoalbuminuric patients. Twenty-five percent of patients in the normoalbuminuric group progressed to the microalbuminuric group one year later. The urinary type IV collagen excretion was increased except in only two patients. On the other hand, other normoalbuminuric patients showed a decrease of urinary type IV collagen excretion one year later. In about half of the normoalbuminuric patients, the levels of urinary type IV collagen returned to the normal range. Recently, Takizawa et al. (16) reported the 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 indicated ongoing alteration in the turnover of glomerular and tubular type IV collagen in the diabetic kidney. It is concluded that the serial measurement of urinary type IV collagen can be a useful marker for detecting renal injury in diabetes.

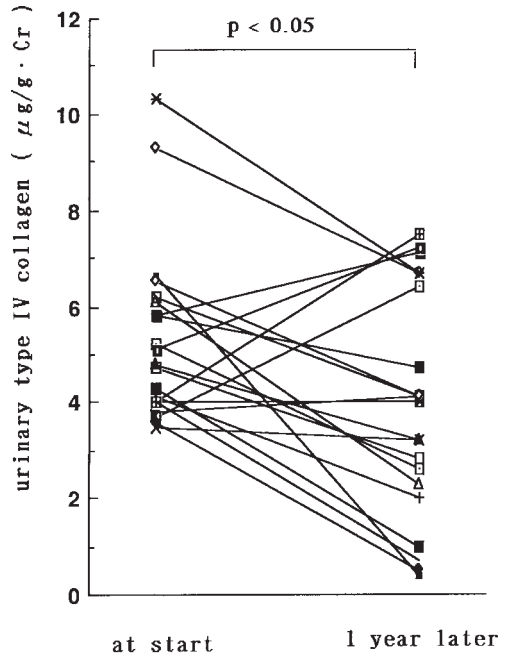

Fig. 4. Alteration of urinary albumin levels in patients that stayed within the normoalbuminuric group one year later.

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