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Serum and Urinary Type IV Collagen Concentrations in the Assessment of Diabetic Microangiopathy

Nazifa BANU, Hitoshi HARA*, Genshi EGUSA and Michio YAMAKIDO

Second Department of Internal Medicine, Hiroshima University School of Medicine, 1-2-3, Kasumi, Minami-ku, Hiroshima 734, Japan

ABSTRACT

We investigated the role of measurement of serum and urinary type IV collagen (IV-C) levels in monitoring diabetic microangiopathy. Furthermore, we compared these levels in diabetic nephropathy and non-diabetic renal disease (NDRD). A one-step sandwich enzyme immunoassay was used to measure IV-C levels in 82 diabetic patients, 33 NDRD patients and 20 healthy non-diabetic control subjects. The diabetic patients were classified into four groups according to urinary albumin / creatinine index (ACI) (mg/g) and serum creatinine (s-Cr) (mg/dl) : normoalbuminuria (ACI<30), microalbuminuria (ACI 30–300), albuminuria (ACI >300, s-Cr<1.99 mg/dl) and renal insufficiency (s-Cr>1.99 mg/dl). Serum and urinary IV-C levels were significantly elevated even in diabetic patients without clinical evidence of microangiopathy compared with control subjects ($p<0.05$ and $p<0.01$, respectively). Both levels were significantly higher in normoalbuminuric patients than in the control subjects, and in patients with microalbuminuria, albuminuria or renal insufficiency than in normoalbuminuric patients, with significant differences between these groups (serum and urinary IV-C, both $p<0.0001$ by ANOVA). Urinary IV-C and albumin levels were significantly correlated, even in normo- and microalbuminuric patients ($r = 0.55$, $p<0.0001$). Serum IV-C in normoalbuminuric patients rose significantly as the degree of retinopathy progressed from background to proliferative stages ($p<0.05$). Neither serum nor urinary IV-C levels were influenced by glycemic control. Albuminuric diabetic patients (with and without renal insufficiency) had significantly higher levels of serum IV-C compared with those in proteinuric NDRD patients ($p<0.005$), though there was no significant difference in the urinary IV-C level. However, the urinary IV-C / albumin ratio was significantly higher in albuminuric diabetic patients than in proteinuric NDRD patients, even after adjusting for s-Cr and creatinine clearance ($p<0.0001$). In conclusion, we suggest that measured serum and urinary IV-C concentrations may serve as new markers for monitoring the development and progression of diabetic microangiopathy, particularly nephropathy. Furthermore, the measurement of serum IV-C concentrations and urinary IV-C / albumin ratios in diabetic patients may allow diabetic nephropathy and non-diabetic renal disease to be differentiated.

Key words: *Type IV collagen, Diabetic nephropathy, Diabetic retinopathy, Non-diabetic renal disease*

Thickening of the capillary basement membranes in various tissues, especially kidney and retina, is a prominent finding in both insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM), and is considered to be an early event in the pathogenesis of diabetic microangiopathy[4,25,48]. In diabetic nephropathy glomerular basement membrane thickening parallels the degree of mesangial expansion[7,44]. Recent biochemical and immunohistochemical studies in diabetic patients as well as in experimentally diabetic rats have demonstrated an imbalance in structural macromolecular components such as type IV collagen (IV-C), laminin and proteoglycans, in thickened glomerular capillary basement membranes and expanded mesangial areas[2,15,24,31,35,42,49]. The predominant abnormality in these structures is increased accumulation and altered distribution of IV-C, which is the major structural component of basement membranes. Similar abnormalities have also been reported in thickened renal tubular and retinal capillary basement membranes[14,15]. Abnormalities of IV-C metabolism therefore appear to be a central event in the development of diabetic microangiopathy, particularly nephropathy.

* Author to whom correspondence should be directed

Increased IV-C production secondary to the diabetic milieu[12,17] as well as decreased degradation secondary to the structural changes induced by diabetes[30] may both be responsible mechanisms.

IV-C and its fragments, which seems to be released into the circulation as a consequence of normal basement membrane metabolism, can be measured in serum by sandwich enzyme immunoassay (EIA) and specific radioimmunoassays (RIA), respectively[19,32,34]. Previous studies have demonstrated increased serum levels of the 7S domain (7S collagen) and the carboxy-terminal domain (NC1) of IV-C in streptozotocin-diabetic rats[3,18,38]. These increased levels are thought to reflect increased synthesis of basement membrane IV-C throughout the body[3,18,38]. Furthermore, serum levels of IV-C and its fragment 7S collagen were reported to be elevated in diabetic patients with microangiopathy[20,33,46]. In a recent study, urinary excretion of the NC1 domain of IV-C was found to be increased in IDDM patients in the early stage of incipient diabetic nephropathy and it was proposed that this reflected altered diabetic glomerular basement membrane metabolism[47]. For clinical use non-invasive methods of measuring the degree of alteration in the basement membrane metabolism are desirable. We postulate that the measurement of serum and urinary IV-C levels in diabetic patients may be useful in assessing alterations in the basement membrane IV-C metabolism and also for monitoring the development and progression of diabetic microangiopathy. In the present study, we therefore measured serum and urinary IV-C by sandwich EIA in diabetic patients with and without microangiopathy in order to assess its value as a non-invasive marker.

Progressive accumulation of normal constituents of extracellular matrix, for example IV-C, laminin and fibronectin, occurs in the glomerular basement membrane and mesangial matrix in a number of non-diabetic renal diseases (NDRD), including membranous nephropathy, IgA nephropathy and focal glomerulosclerosis[16,23,27,28,45]. Elevated urinary IV-C concentrations may therefore not only indicate diabetic nephropathy, but also suggest the presence of NDRD in diabetic patients. A recent study demonstrated raised serum and urine levels of the NC1 domain of IV-C in patients with glomerulonephritis compared with those in healthy controls[26]. Furthermore, in diabetic patients it is desirable to ascertain whether proteinuria results from diabetic or non-diabetic renal involvement. In order to evaluate whether the determination of serum and urinary IV-C levels is helpful in differentiating diabetic nephropathy and non-diabetic renal disease, we compared these levels in patients with diabetic nephropathy and non-diabetic renal disease.

MATERIALS AND METHODS

Subjects

Eighty-two diabetic patients (69 NIDDM and 13 IDDM; 31 males and 51 females) with a mean (±SD) age of 53 (±13) years (range 25–75 years) and 33 NDRD patients (16 males and 17 females) with a mean (±SD) age of 45 (±17) years (range 14–70 years) were studied. The study was conducted on an inpatient basis. No patient had clinical evidence of liver dysfunction. Diabetic patients with clinical and/or serological signs of connective tissue disorders were excluded. Twenty healthy non-diabetic subjects [mean (±SD) age 55 (±9) years, range 40–69 years] undergoing routine annual medical examinations were recruited as a control group.

The diagnosis of all NDRD was established by renal biopsy. In diabetic patients, the known duration of diabetes, body mass index (BMI), blood pressure (BP) and type of antidiabetic treatment were recorded. Glycemic control was assessed by HbA1C measurements. Twelve NIDDM patients were followed for 8 weeks to study the effect of improved glycemic control on IV-C levels. The grade of retinopathy was determined by ophthalmologists using ophthalmoscopy and / or fluorescein angiography, and classified as without retinopathy or with retinopathy including background and proliferative retinopathy. The degree of nephropathy was assessed by urinary albumin / creatinine index (ACI) and serum creatinine values. The diabetic patients were divided into four groups according to the degree of nephropathy: (1) normoalbuminuria: ACI<30 mg/g, (2) microalbuminuria: ACI 30–300 mg/g, (3) albuminuria: ACI>300 mg/g but serum creatinine level<1.99 mg/dl, (4) renal insufficiency: serum creatinine level >1.99 mg/dl. The ACI was calculated as urinary albumin concentration (mg/L) / urinary creatinine concentration (g/L)[41].

Blood and urinary samples

Blood and urine samples for measurement of IV-C were collected on the same day. In diabetic patients serum IV-C was measured in fasting blood samples. Urinary IV-C and albumin were measured in 24 hour urine collections. The patients who were followed for 8 weeks had a total of three blood and urine examinations over this period (two during their inpatient stay and a final measurement in our outpatient-clinic within 2 weeks of discharge). In these patients, first void early morning urine samples were used.

Serum for IV-C measurement was obtained from venous blood after clotting and centrifuging blood samples for 15 min at 1,800×g at 4°C. The original whole urine was concentrated 100-fold before IV-C measurement, because in most urine samples the levels were below the detection limit of the assay. Concentration was performed with

the use of 150 g/L polyethylene glycol (PEG) – 4000 in 0.2M Tris-HCL, pH 7.6 (containing 6.15 mM sodium azide), in the presence of 0.5 g/L γ-globulin, as we described previously[1]. Serum and PEG-concentrated urine samples were stored at –70°C until analysis of IV-C.

Laboratory methods

IV-C concentration was measured in duplicate by a one-step sandwich EIA using the Panassay IV-C kit (Fuji Chemical Industries Ltd., Toyama, Japan)[34]. In this assay two distinct monoclonal antibodies were used, one (clone 4H12) against the 7S domain and the other (clone 1D3) against the central triple-helical domain of IV-C molecule. Briefly, 50 μl of serum or PEG-concentrated urine sample or standard IV-C solution was incubated with a polystyrene ball coated with mouse anti-human IV-C monoclonal antibody (clone 4H12) and 300μl of Fab' (clone 1D3)-peroxidase (0.8 mg/L) in 10 mM Na-phosphate buffer, pH 7.0 for 60 min at room temperature. After stopping the immunoreaction with 1ml of 10 mM Na-phosphate buffer, pH 7.0 containing 0.1M NaCl, the polystyrene ball was washed 5 times with 3ml of the same buffer. Subsequently, the polystyrene ball was incubated with 300μl of 3,3',5,5'-tetramethylbenzidine (0.134 g/L) and 100μl of hydrogen peroxide (0.15 g/L) in 100 mM acetate buffer (pH 5.5) for 30 min at room temperature. The enzyme reaction was stopped by adding 1 ml 1.33 N sulphuric acid, and the absorbance at 450 nm was measured by a double-beam spectophotometer (Model-2000; Hitachi, Tokyo, Japan) to calculate IV-C concentration. IV-C measurement from PEG-concentrated urine samples showed good recovery (102.6 ± 1.0%) and reproducibility [intra- and interassay coefficients of variation (CVs) 5.5 ± 0.7% and 4.3 ± 1.7%, respectively]. The intra- and interassay CVs for serum IV-C were 3.2 ± 0.5% and 5.0 ± 1.0%, respectively.

The blood glucose was measured by a glucose analyzer and HbA1C by HPLC (Glucose AUTO & STAT™ and AUTO A1C, respectively; Kyoto Daiichi Chemical Co. Ltd., Japan). Serum cholesterol and triglyceride were determined enzymatically (EA test T-CHO600, Triglyzyme®-600; Eiken Chemical Co.Ltd., Tokyo, Japan). Serum and urinary creatinine were measured by an enzymatic method using a commercial kit (AR CRE-N; Mizuho Medy, Tosu, Japan). Urinary albumin was measured by a latex turbidimetric immunoassay using mouse anti-human albumin antiserum-coated latex (Eiken Chemical Co.Ltd., Tokyo, Japan) and immunochemistry analyzer (Model LA-1000; Analytical instruments, Tokyo, Japan)[22,43]. This assay has an intra- and interassay CV of 4.2 ± 1.1% and 4.5 ± 0.7% respectively at concentrations of 1.1–17.0 mg/L. Before quantitation, the urine was screened with a semi-quantitative test strip (Uristix; Ames Division, Miles-Sankyo Co. Ltd., Tokyo, Japan) to indicate those samples with excess albumin which required dilution.

The molecular size distribution of IV-C in the serum and PEG-concentrated urine samples was determined by Western blotting of immunoprecipitated antigens from these samples. Immunoprecipitation was carried out using rabbit anti-human IV-C polyclonal antibody (Chemicon International Inc.; Temecula, CA) and goat anti-rabbit IgG as primary and secondary antibodies, respectively. The immunoprecipitate from the serum, urine and calibrator human placental IV-C solution (500 μg/L), together with the high molecular weight calibration kit proteins (Pharmacia LKB Biotechnology, Uppsala, Sweden) were then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a SDS-polyacrylamide gel with a concentration gradient of 4–20% (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan). After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) using an ISS (integrated separation systems) Semidry Electroblotter (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan). The membrane was incubated with mouse anti-human IV-C monoclonal antibody (clone 4H12) (Fuji Chemical Ind. Ltd., Toyama, Japan), and then with HRP-conjugated rabbit anti-mouse IgG, as described previously[40]. Immunoreactive protein bands were visualized by staining with 0.28 mM 3,3'-diaminobenzidine (Dojindo Lab., Kumamoto, Japan) and 1.63 mM H_2O_2. Finally, the staining pattern of collagenous proteins was intensified by the Gold-Sulphide-Silver(GOSS) method of Iida et al[21].

Statistical analysis

Statistical analyses were performed using Stat view (Abacus Concepts Inc., CA, USA) statistical software on an Apple Macintosh IIci computer (Apple computer Inc., Cupertino, CA, USA). All data are presented as mean ± SEM unless specified otherwise. Analysis of variance (ANOVA) was performed to compare multiple groups. When results were statistically significant by ANOVA, the significance of differences between any two groups was determined by two-tailed Student's t-test. A probability of $p<0.05$ was considered as statistically significant. Relationships between variables were assessed by Spearman rank correlation or by linear regression analysis.

RESULTS

The clinical characteristics of the diabetic patients studied are shown in Table 1. The four patient groups did not differ significantly in age, duration of diabetes or BMI. Compared with the normoalbuminuric patients, patients with micro-

Table 1. Clinical characteristics of the diabetic patients studied

	Diabetic patient groups			
	Normoalbuminuria	Microalbuminuria	Albuminuria	Renal insufficiency
n (M/F)	39 (12/27)	26 (13/13)	12 (4/8)	5 (2/3)
NIDDM/IDDM (n)	30/9	22/4	12/0	5/0
Age (year)	49±14	56±13	58±12	61±1
Known duration of diabetes (year)	15±10	18±7	18±5	17±9
BMI (kg/m^2)	22.0±3.2	22.1±3.0	21.9±3.3	24.1±1.7
Prevalence of retinopathy (%)*	39/20/41	8/30/62	0/25/75	0/0/100
Antidiabetic treatment (%)¶	18/18/49/15	4/19/69/8	8/25/59/8	20/0/60/20
Systolic BP (mmHg)	132±19	135±20	156±24[b,g]	178±22[a,f]
Diastolic BP (mmHg)	78±11	76±10	89±10[c,g]	94±4[c,f]
Fasting blood glucose (mg/dl)	160±41	171±52	146±43	116±18[c,h]
HbA1c (%)	8.5±1.6	8.6±1.7	7.5±2.0	7.0±1.7[d]
Serum creatinine (mg/dl)	0.70±0.20	0.70±0.21	1.19±0.40[a,e]	3.50±1.01[a,e,i]
Urinary albumin (mg/L)	9.7±6.9	83.7±89.7[a]	495.9±284.6[a,e]	653.2±167.7[a,e]

Data given as mean±SD if not otherwise indicated. BP, blood pressure; BMI, body mass index.
* Retinopathy is shown as normal retina/background retinopathy/proliferative retinopathy.
¶ Antidiabetic treatment is shown as diet only/oral hypoglycemic agents/insulin/oral hypoglycemic agents+insulin.
[a] $p<0.0001$, [b] $p<0.001$, [c] $p<0.01$, [d] $p<0.05$ vs nomoalbuminuria. [e] $p<0.0001$, [f] $p<0.001$, [g] $p<0.01$, [h] $p<0.05$ vs microalbuminuria. [i] $p<0.0001$ vs albuminuria.

albuminuria, albuminuria and renal insufficiency had a higher prevalence of background or proliferative retinopathy and were more often on insulin therapy. The albuminuric patients and patients with renal insufficiency had significantly higher systolic and diastolic BP (sBP, dBP) compared with the normo- and microalbuminuric groups. No significant differences were found in fasting blood glucose and HbA1C levels among the normoalbuminuric, microalbuminuric and albuminuric groups, but these values were somewhat lower in the patients with renal insufficiency ($p<0.05$).

Serum and urinary IV-C levels were significantly elevated in diabetic patients with microangiopathy compared with control subjects (138.8±4.5 vs 85.0±2.8μg/L, $p<0.0001$ and 77.8±5.4 vs 21.4±2.7 μg/L, $p<0.0001$, respectively) and even in those without clinical evidence of microangiopathy (97.2±5.0 vs 85.0±2.8 μg/L, $p<0.05$ and 33.8±3.3 vs 21.4±2.7 μg/L, $p<0.01$, respectively); the levels in diabetic patients showed no sex difference and did not correlate with BMI.

Serum IV-C levels increased slightly with age in the diabetic patients and were significantly higher ($p<0.01$) in patients ≧60 years old compared with <40; whereas in the control subjects serum IV-C levels were not correlated with age. Urinary IV-C levels in both the control and diabetic subjects did not show any significant change with age. In diabetic patients with a known duration of diabetes of <5, 5–9, 10–14, 15–19 and ≧20 years, serum IV-C levels were 94.6±7.3, 137.9±16.4, 124.0±8.6, 143.2±7.0, 137.6±7.0 μg/L, and urinary IV-C levels were 34.3±5.4, 65.6±14.4, 61.9±8.5, 62.2±9.6, 92.6±9.8 μg/L, respectively. By ANOVA, both serum and urinary IV-C showed statistically significant differences between these groups ($p<0.05$ and $p<0.01$, respectively).

Fig. 1 shows serum and urinary IV-C levels in the control subjects and in the four groups of diabetic patients. There were significantly higher levels of serum and urinary IV-C in normoalbuminuric diabetic patients (111.7±4.6 and 44.5±3.1 μg/L) compared with control subjects (85.0±2.8 and 21.4±2.7 μg/L). These levels further increased in patients with microalbuminuria (136.9±7.0 and 70.9±6.7 μg/L), albuminuria (166.7±9.6 and 113.0±11.5 μg/L) and renal insufficiency (163.5±15.6 and 156.8±19.1 μg/L) compared to those with normoalbuminuria; ANOVA confirmed significant differences between these groups (serum and urinary IV-C, both $p<0.0001$). The small number of IDDM patients were found only in the normo- and microalbuminuric groups, and their serum and urinary IV-C levels did not differ significantly compared with the respective groups of NIDDM patients (data not shown). Even in normo- and microalbuminuric patients, linear regression analysis demonstrated significant positive correlation between

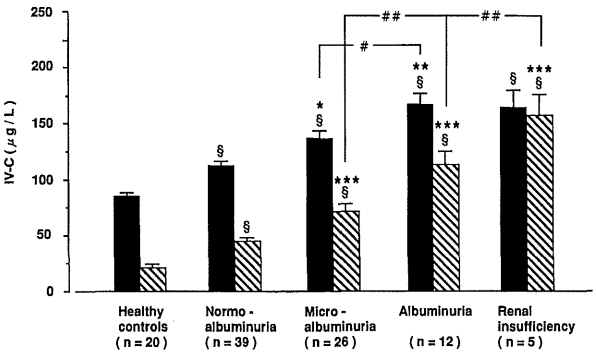

Fig. 1. Serum (■) and urinary (▨) IV-C levels in control subjects and diabetic patients with different degrees of nephropathy. Values are mean ± SEM. §p<0.0001, control subjects vs all four groups of diabetic patients; ***p<0.0001, normoalbuminuria vs all other groups of diabetic patients; *p<0.005 and **p<0.001, normoalbuminuria vs microalbuminuria and albuminuria respectively; #p<0.05, microalbuminuria vs albuminuria; ##p<0.001, microalbuminuria vs albuminuria and albuminuria vs renal insufficiency.

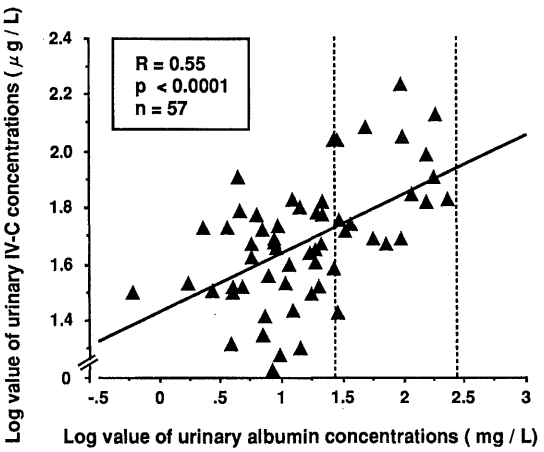

Fig. 2. Relationship between urinary IV-C and albumin levels in normo- and microalbuminuric diabetic patients. A logarithmic transformation was used to normalise the distribution of urinary albumin and IV-C data. The solid line indicates linear regression and dotted lines represent the upper limit of normal for urinary albumin (log transformed value 1.48) and for microalbuminuria (log transformed value 2.48).

urinary IV-C and albumin levels ($r = 0.55$, $p<0.0001$; Fig. 2). In those patients with ACI <15, 16–30, 31–90, 91–150 and 151–300 mg/g, urinary IV-C levels were also found to be proportionately elevated ($p<0.001$, ANOVA); however, using linear regression and the same ACI groupings, no correlation was noted between the serum IV-C and urinary albumin levels.

When the four diabetic patient groups were considered together, serum and urinary IV-C

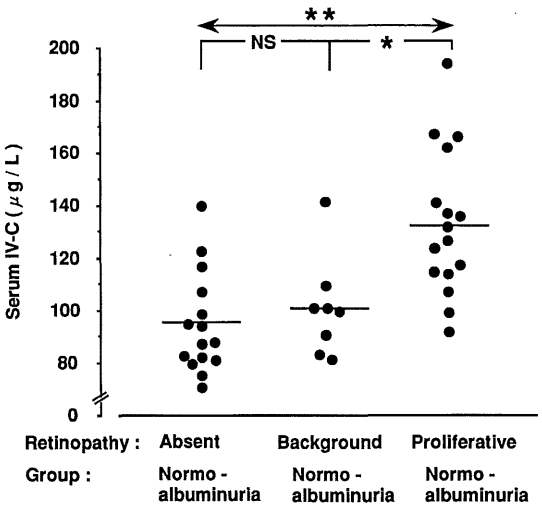

Fig. 3. Serum levels of IV-C in normoalbuminuric diabetic patients with different grades of retinopathy compared with those without retinopathy. Horizontal lines represent mean values. NS, not significant; **p<0.001, no retinopathy vs proliferative retinopathy; *p<0.05, background retinopathy vs proliferative retinopathy.

showed significant elevations as the degree of retinopathy progressed from background to proliferative stages ($p<0.01$ and $p<0.05$, respectively by ANOVA). This observation remained statistically significant only for serum IV-C even in normoalbuminuric patients (Fig. 3).

Table 2 shows that the serum and urinary IV-C levels were not influenced by either blood glucose or HbA1C levels. Improved glycemic control, as indicated by a decrease in mean fasting blood glucose from 231.5 ± 14.8 mg/dl to 124.7 ± 7.9 mg/dl and mean HbA1C from 9.8 ± 0.3% to 6.8 ± 0.3% during 8 weeks of follow-up, did not alter the levels of serum and urinary IV-C (Fig. 4). Furthermore, there was no relationship between the type of antidiabetic treatment and serum or urinary IV-C levels.

Table 2, which represents the associations of serum and urinary IV-C levels with other variables in the diabetic patients taken as a whole, shows that both serum and urinary IV-C levels were significantly correlated with sBP, and urinary IV-C also with dBP. There was no correlation of serum and urinary IV-C levels with fasting serum cholesterol level, but there was a significant correlation between serum IV-C and fasting serum triglyceride levels. Serum and urinary IV-C were significantly correlated with serum creatinine. However, in patients with nephropathy (all patients apart from those with normoalbuminuria) serum IV-C did not show any intimate relationship with serum creatinine, serum β2-microglobulin or creatinine clearance; though in these patients correlations between urinary IV-C and

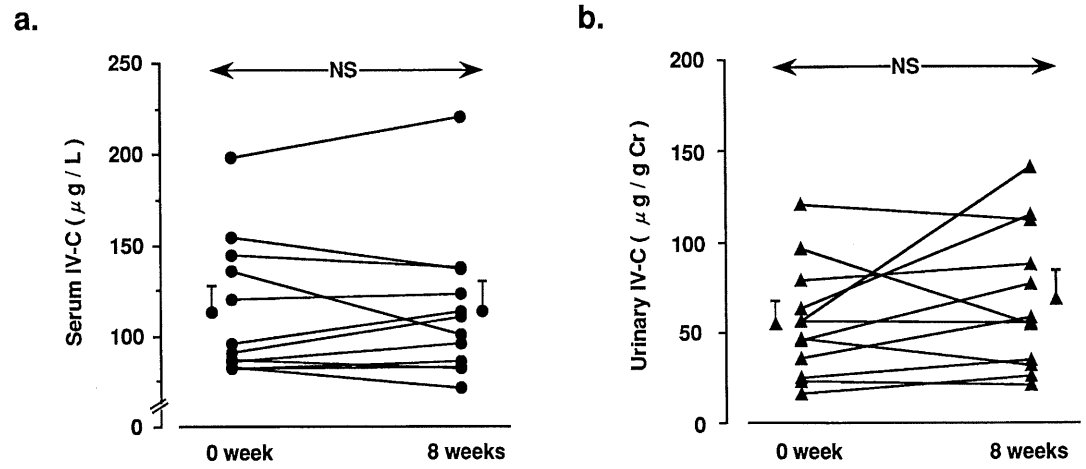

Fig. 4. Serum IV-C (a) and urinary IV-C (b) levels in the 12 diabetic patients before and after improvement of glycemic control, during 8 weeks of follow-up. Urinary IV-C levels (μg/L) are expressed in relation to creatinine (g/L) to exclude the influence of urine volume, as measured in first void early morning urine samples. NS, not significant.

Table 2. Association of serum and urinary IV-C levels with other variables, assessed by Spearman rank correlation

	Serum IV-C		Urinary IV-C	
	Correlation coefficient	p value	Correlation coefficient	p value
HbA1c (%)	–0.19	NS	–0.2	NS
Fasting blood glucose (mg/dl)	–0.09	NS	–0.2	NS
Systolic BP (mmHg)	0.30	<0.01	0.30	<0.01
Diastolic BP (mmHg)	0.22	NS	0.26	<0.05
Fasting serum cholesterol (mg/dl)	0.24	NS	0.17	NS
Fasting serum triglyceride (mg/dl)	0.37	<0.01	0.25	NS
Serum creatinine (mg/dl)	0.35	<0.005	0.32	<0.005

BP, blood pressure; NS, not significant.

these parameters of renal function were present (data not shown). The urinary IV-C level did not show any significant association with the serum IV-C level in patients with or without nephropathy.

Taken as a whole, NDRD patients had significantly elevated serum and urinary IV-C levels compared with control subjects (113.2 ± 8.6 vs 85.0 ± 2.8 μg/L, $p<0.01$ and 115.5 ± 10.8 vs 21.4 ± 2.7 μg/L, $p<0.0001$, respectively). Table 3 demonstrates the serum and urinary IV-C levels in various types of NDRD. Although these levels were apparently higher in IgA nephropathy and lupus nephritis than in other NDRD groups, statistical significance was not achieved on account of the very small group sizes. In these patients, neither serum nor urinary IV-C levels showed any significant correlation with serum creatinine and creatinine clearance values. In NDRD patients, unlike diabetic patients, there was no correlation between urinary IV-C and albumin levels.

There was a significantly lower level of serum IV-C in proteinuric NDRD patients compared with albuminuric diabetic patients (with and without renal insufficiency) (113.2 ± 8.6 vs 165.7 ± 7.9 μg/L, $p<0.005$), whereas there was no significant difference in urinary IV-C levels between these two groups (Fig. 5a & 5b). However, the ratio of urinary IV-C to albumin was significantly lower in proteinuric NDRD patients than in albuminuric diabetic patients, even after adjusting for serum creatinine and creatinine clearance (120 ± 20 vs 310 ± 50, $p<0.0001$) (Fig. 5c).

Fig. 6 shows the results of Western blotting under reducing condition. The immunoreaction of the blotted antigen with a monoclonal antibody (clone 4H12) which was used in the IV-C assay system, revealed a single band of α(IV) monomer in both serum and urine, corresponding to a mo-

Table 3. Serum and urinary IV-C levels in various types of non-diabetic renal diseases

	Serum creatinine (mg/dl)	Serum IV-C (μg/L)	Urinary albumin (g/L)	Urinary IV-C (μg/L)
Minimal change GN (nephrotic phase) (n=3)	0.71±0.09	92.7±20.0	2.5±1.2	72.9±15.5
Mesangial proliferative GN (IgA nephropathy) (n=11)	4.59±1.20	122.5±20.3	1.6±0.5	121.8±13.2
Non IgA mesangial proliferative GN (n=5)	1.10±0.20	117.5±23.8	1.5±0.7	112.1±28.9
Membranous GN (n=6)	1.80±1.01	90.8±11.9	1.6±0.4	95.6±29.2
Mesangiocapillary GN (n=1)	0.93	144.4	1.0	58.5
Sclerosing GN (n=2)	5.19±2.01	119.9±42.3	2.8±1.9	112.4±30.4
Lupus nephritis (n=3)	0.90±0.20	142.7±21.7	1.4±0.4	218.6±48.3
Amyloidosis (n=1)	6.79	72.7	0.27	81.9

All values given as mean±SEM. GN, glomerulonephritis.

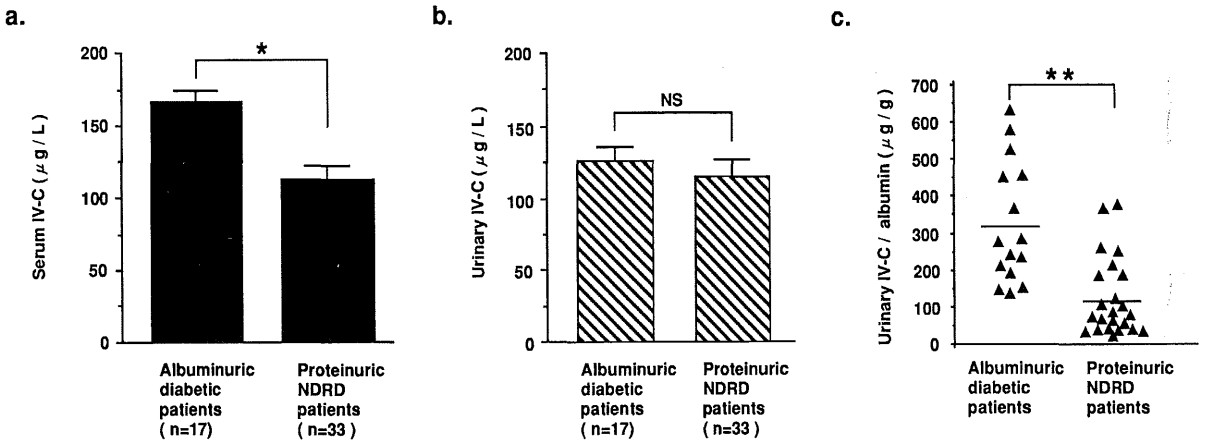

Fig. 5. Serum IV-C (a), urinary IV-C (b) levels, and the urinary IV-C / albumin ratio (c) in proteinuric NDRD patients compared with albuminuric diabetic patients. In (a) and (b) results are mean±SEM. In (c) ratios are shown after adjusting serum creatinine and creatinine clearance values of two patient groups. NS, not significant; *p<0.005 and **p<0.0001, albuminuric diabetic patients vs proteinuric NDRD patients.

lecular mass of approximately 140 kDa. Serum and urinary α(IV) monomeric fragments in diabetic and NDRD patients had a similar mobility to calibrator human placental IV-C.

DISCUSSION

A number of recent studies have demonstrated elevated levels of 7S collagen and type IV collagen in the serum of diabetic patients with microangiopathy and a significant difference in its concentration between those with and without microangiopathy[20,33,46]. Although the NC1 domain of type IV collagen has been found to be elevated in the serum of streptozotocin-diabetic rats[3], some studies have shown no rise in serum NC1 levels in diabetic nephropathy and vasculopathy[13,47]. Torffvit et al[47], who demonstrated increased urinary NC1 excretion in IDDM patients in incipient diabetic nephropathy, could not detect any rise in serum NC1 in the same patients. Although the results of most previous studies have been similar to those found here, the source of some of the variation was probably due to the use of different radioimmunochemical methods for the measurement of IV-C and its fragments. The one-step sandwich EIA method used in the present study is highly sensitive and specific for the detection of this antigen in serum[32,34]. The sensitivity of the assay, which was not sufficient for detection of IV-C in original whole urine except in a few cases of advanced diabetic nephropathy[33], was increased in this study by PEG-based 100-fold concentration of urine, which showed good recovery and reproducibility for the measurement of urinary IV-C in our previous study[1].

In this study, serum and urinary concentrations of IV-C were measured at different stages of diabetic microangiopathy, particularly nephropathy, and were compared with measurements in pa-

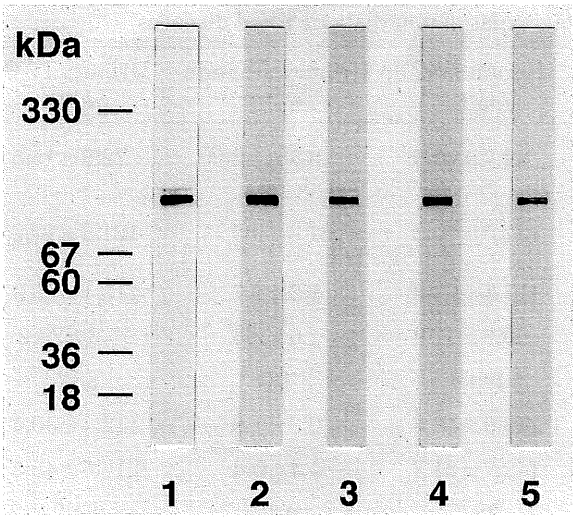

Fig. 6. Western blotting of immunoprecipitate from calibrator human placental IV-C (lane 1), serum and PEG-concentrated urine samples of a patient with diabetic nephropathy (lanes 2 & 3, respectively) and non-diabetic renal disease (lanes 4 & 5, respectively).
Molecular weight standards are shown in kilodaltons.

tients with various non-diabetic renal diseases. Both serum and urinary IV-C levels were distinctly elevated even in diabetic patients without clinical evidence of microangiopathy, compared with control subjects, and the degree of elevation increased with progression of nephropathy and retinopathy. These observations confirmed our speculation that determination of serum and urinary IV-C levels in diabetic patients may be important in the early detection and follow-up of the progression of microangiopathy.

The significant correlations of urinary IV-C level with the level of urinary albumin and various parameters of renal function such as serum creatinine and creatinine clearance values indicate that elevation of urinary IV-C level is associated with progression of diabetic nephropathy. Moreover, the elevated urinary IV-C levels found even in normoalbuminuric diabetic patients, and its proportionate rise with increase in ACI in normo- and microalbuminuric diabetic patients, indicate that urinary IV-C concentrations may also be a useful indicator of incipient renal damage. Although a significant relationship was found between urinary IV-C levels and progression of retinopathy in the diabetic population of this study, this observation may however be the effect of concurrent nephropathy in patients with advanced retinopathy, since the patients with clinical evidence of nephropathy had a higher prevalence of proliferative retinopathy (Table 1), and a previous study reported a higher concordance rate between renal and retinal lesions in diabetic patients with advanced retinopathy[8]. On the other hand, the raised serum IV-C found in association with the progression of retinopathy even in normoalbuminuric patients indicates that not only the progression of nephropathy but also of retinopathy might have an influence on serum IV-C levels.

In a recent study (a report in abstract form, in Diabetes 40: 263A, 1991), Matsumoto et al found a weak negative correlation between fructosamine and serum IV-C levels; this was thought to be the result of suppressed biosynthesis of IV-C in poorly controlled patients. In our study, glycemic control as assessed by HbA1C did not correlate with either serum or urinary IV-C levels. This is consistent with the findings of Tomono et al[46]. Furthermore, the absence of changes in serum and urinary IV-C levels with improvement of glycemic control during 8 weeks of follow-up suggests that short term changes in glycemic control have no effect on serum and urinary IV-C levels.

In the present study, albuminuric diabetic patients with or without renal insufficiency had significantly higher sBP and dBP compared with normo- and microalbuminuric patients. The significant correlations observed between IV-C levels and BP may therefore partly be attributable to hypertension, which was present in association with diabetic nephropathy. Previous studies have demonstrated raised serum triglyceride levels in diabetic patients with nephropathy[6] and a major association between urinary albumin excretion and fasting serum triglycerides[11]. In our study, we observed similar results (data not shown). However, urinary IV-C levels which were raised proportionately to the degree of progression of diabetic nephropathy, and which were significantly correlated with urinary albumin levels, did not show any association with serum triglycerides; whereas we found a significant association between serum IV-C and triglycerides. The significance of this observation is therefore uncertain.

The four groups of diabetic patients studied here did not differ significantly in the duration of diabetes; however, an increased prevalence of microvascular complications was found with the increase in length of duration. A significant rise in serum and urinary IV-C levels found after 5 years of the onset of diabetes may be the result of the increased incidence or prevalence and severity of diabetic microangiopathy associated with increased length of duration. Higher serum IV-C concentrations found in diabetic patients of $\geqq 60$ years of age compared with those of <40 years of age may also be the effect of diabetic microangiopathy, because the older patients had a higher prevalence of microangiopathy. Although the control subjects did not show any correlation between age and serum IV-C concentration, the possibility of a significant age effect can not be

excluded because subjects under 40 were not studied.

Recently, using the technique of molecular sieving chromatography, we have found that sandwich EIA detects a single peak of >500,000 Mr IV-C in PEG-concentrated urine[1]. Using the same method, a previous study also demonstrated a high molecular weight IV-C in serum[32]. In the present study, immunoblotting with a monoclonal antibody, which was used in the IV-C assay system, revealed that serum and urinary IV-C in diabetic and NDRD patients has a high molecular weight similar to human placental IV-C. These results indicate that serum and urinary IV-C, measured by sandwich EIA, is a whole molecule and not a degradation product of pre-existing IV-C. Measurement of IV-C levels by EIA probably reflects their increased synthesis or decreased degradation. Several recent in vivo and in vitro studies have suggested that the increased IV-C accumulation in glomerular and tubular basement membranes, and in mesangial matrix found in diabetic nephropathy, is due to increased synthesis[5,9,12,18,29,36,37,50] or decreased degradation[10,39].

Elevated serum IV-C levels in diabetic patients are probably not caused by impaired renal excretion, because they did not correlate with serum creatinine or creatinine clearance in patients with nephropathy. Furthermore, the finding of high serum IV-C levels even in normoalbuminuric patients excludes this possibility. Raised serum IV-C concentrations most likely originate from altered basement membrane IV-C metabolism of various tissues involved in diabetic microangiopathy. The origin of urinary IV-C in diabetic patients is unclear. Urinary IV-C is a high molecular weight protein. Nevertheless, elevated levels were found in patients with incipient nephropathy and even in those without evidence of nephropathy who probably have a normal glomerular filtration barrier. Alterations of glomerular and tubular basement membrane metabolism rather than glomerular filtration dysfunction may be the origin of raised urinary IV-C in these patients. However, these patients showed a significant correlation between urinary IV-C and albumin, so the possibility of increased glomerular filtration of IV-C in the early stage of nephropathy cannot be excluded. In the advanced stage of diabetic nephropathy elevated urinary IV-C could, however, reflect a disorder of the glomerular filtration barrier together with altered glomerular and tubular basement membrane metabolism, although there was no correlation between serum and urinary IV-C in patients with clinical evidence of nephropathy.

In the present study we found elevated serum and urinary IV-C levels in various NDRD, the levels differing according to the intrinsic type of renal disease. These findings are consistent with those of Keller et al[26]. In our study, the NDRD patients who were receiving our inpatient treatment were included and could be considered to have active rather than chronic renal disease because they had either a deterioration of renal function or an indication for renal biopsy or were being treated with immunosuppressive agents. Furthermore, the NDRD patients studied here also had greater proteinuria and worse renal function than albuminuric diabetic patients. However, interestingly, the proteinuric NDRD patients as a whole had lower serum IV-C levels than the albuminuric diabetic patients. Furthermore, even after adjusting for serum creatinine and creatinine clearance, there was a significant difference in the urinary IV-C / albumin ratio between the proteinuric NDRD patients and albuminuric diabetic patients. In diabetic patients it is desirable to ascertain whether proteinuria results from diabetic or non-diabetic renal involvement. However, this is a rather difficult task for a clinician, without histological findings of renal biopsy, particularly when diabetic patients are presented with albuminuria in the presence or absence of mild retinopathy together with a short medical history of NIDDM. The findings of our study suggest that a low serum IV-C level together with a low urinary IV-C / albumin ratio in these diabetic cases may indicate the presence of NDRD rather than diabetic nephropathy. In NDRD patients elevated serum IV-C most likely originates from the kidney, whereas in diabetic patients with nephropathy it probably originates from the basement membranes of various tissues involved in microangiopathy rather than from the kidney alone. This may explain the lower level of serum IV-C in NDRD patients. In NDRD patients urinary IV-C level was independent of renal function, whereas in albuminuric diabetic patients it was associated with deteriorating renal function.

We conclude that the measurement of serum and urinary IV-C concentrations may be a useful new marker for monitoring the development and progression of diabetic microangiopathy, particularly nephropathy. From the results of the present study, urinary IV-C appears to be a more sensitive marker of early diabetic nephropathy than serum IV-C. Assessment of the degree of alteration in glomerular extracellular matrices and tubular basement membrane in diabetic patients may also be possible by measuring urinary IV-C. In addition, determination of the serum IV-C and urinary IV-C / albumin ratio may allow diabetic nephropathy and non-diabetic renal disease to be differentiated.

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