Increased urinary fibronectin excretion in type II diabetic patients with microalbuminuria

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A series of recent observations have shown raised levels of plasma fibronectin (FN), an \( \alpha_2 \)-glycoprotein produced by vascular endothelia, in diabetic patients with retinopathy and overt nephropathy. However, there are no available data on urinary FN and behavior of its excretion in patients affected by diabetic nephropathy characterized by the presence of microalbuminuria. The main purpose of the present study was to investigate whether the urinary excretion of FN (U-FN) is associated with early diabetic nephropathy and other diabetic complications. Fifty-nine diabetic inpatients classified as type II and 15 age-matched control subjects were included in this study. The amount of U-FN, assessed as mg/g creatinine/24 h, was significantly greater in the patients as a group (348.1 ± 48.3), than in the controls (108.6 ± 22.7, p < 0.01). Although patients with overt proteinuria showed an extremely high level of U-FN (1080.5 ± 184.0; range 216.1 – 1726.8), mean U-FN tended to be higher in the group with microalbuminuria (262.4 ± 21.9; range 101.9 – 591.9) than in the group without it (188.1 ± 34.3; range 19.4 – 582.4, p < 0.08). In patients who did not have retinopathy and neuropathy, the U-FN was significantly higher in the group with microalbuminuria (222.5 ± 28.5) than in the group without it (116.1 ± 22.6, p < 0.01). A highly significant negative correlation existed between endogenous creatinine clearance values and the amounts of U-FN in the patients (r = –0.642, p < 0.01), while there was no such relationship in the controls (r = 0.167, p = NS). These results not only suggest that U-FN could be a marker for early diabetic nephropathy, but also imply that increment in U-FN is associated with the other complications affected by diabetes considered in this study.


Key words: urinary fibronectin excretion, diabetes mellitus, diabetic nephropathy, microalbuminuria

Introduction

Fibronectin (FN) is an adhesive \( \alpha_2 \)-glycoprotein present in a fibrillar form on the cell surface and in a soluble form in plasma [1, 2]. FN in circulating blood has been thought to originate from vascular endothelial cells, since both in vitro and in vivo studies revealed that endothelial cells produce FN [3–5]. Among its biologic properties, this glycoprotein has a role in erythrocyte or platelet adhesion to subendothelial collagen and in reducing erythrocyte deformability [6, 7].

Recently, accumulating evidence has suggested an increased level of plasma FN in diabetic patients, particularly in the presence of microvascular complications [8–10]. Further, increase in plasma FN has been described in patients with diabetic overt nephropathy [9, 11, 12] and in nondiabetic subjects affected by nephrotic syndrome [13, 14]. In this context, De Giorgio and his colleagues [15] reported a raised level of plasma FN in diabetic patients with microalbuminuria when compared to those without it. They also demonstrated a significant positive correlation between plasma levels of FN and urinary albumin excretion rate. These findings lead to the plausible hypothesis that an increased level of plasma FN is related to microalbuminuria in diabetic patients.

On one hand, immunohistochemical studies disclosed that FN is produced by renal mesangial cells [16]. It was also found in these studies that FN in these cells is increased proportionately in the disease states characterized by mesangial expansion including diabetic nodules [17–19]. Increased urinary excretion of FN has been demonstrated in human diabetes [20]. Additionally, biochemical evidence suggests that FN detected in human urine, from both normal and diseased subjects, is of renal origin [20]. Thus, these observations may indicate the importance of urinary FN as a marker of diabetic
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nephropathy, as a consequence of renal involvement. To date, however, there are no detailed data on the urinary excretion rate of FN in diabetic patients at different stages of the disease.

Accordingly, the main purpose of the present study was to evaluate the urinary excretion of FN (U-FN) in long-standing type II diabetics with and without diabetic nephropathy.

Subjects and Methods

Subject

Fifty-nine inpatients (27 men and 32 women) with ages ranging from 26 to 80 years (mean age, 58) were included in this study and comprised randomly selected diabetic patients admitted for control of their diabetes to the Third Department of Internal Medicine, Fukushima Medical College. Patients with liver diseases, neoplasms, collagen diseases, infectious diseases, or prostatic hyper trophy were precluded.

Based on the amounts of urinary albumin, these patients were divided into three groups (G), G-O without microalbuminuria (<15 mg/g creatinine (cr)/24 h) [21], G-I with microalbuminuria (≥15 mg/g cr/24 h), and G-II with overt proteinuria (>0.5 g protein/24 h). The clinical characteristics, e.g., age, sex, duration of diabetes, presence or absence of diabetic retinopathy and neuropathy, and glycosylated hemoglobin A1c (HbA1c), of these patients are shown in Table 1. The severity of retinopathy was estimated immediately after admission with a fluorescent technique. Diabetic neuropathy was assessed by the extent of loss of vibratory sensation in the feet. In G-O, 55% were affected by neither retinopathy nor neuropathy; however, 74% and 75% were found to have retinopathy, or neuropathy, or both, in G-I and G-II, respectively (Table 2). Fifteen healthy, non-diabetic individuals (10 men and 5 women) with ages ranging from 18 to 78 years (mean age, 52) were used as normal controls. There were no statistically significant differences in age, duration of diabetes, or HbA1c levels among the different groups of patients (Table 1).

None of the patients or the normal controls were obese (<20% of their standardized body weight). All of the normal controls were in good health and none was under treatment. Of the 59 diabetic patients, 29 were being treated with diet alone, 22 with oral hypoglycemic agents and diet, and 8 with insulin (mean dosage, 23 U NPH). None of the patients was undergoing any form of associated therapy with anticoagulants, antihypertensives or vasodilating agents.

All the patients and normal controls were placed on optimum caloric diets (25 cal/standardized body weight per day) containing 150 mmol of sodium per day for at least 1 week before the study was initiated. During the equilibration period, basal assessment of renal functions (endogenous Cr clearance (Ccr), rate of phenolsulfonphthalein (PSP) excretion 15 min after intravenous injection, and Fishberg’s concentration test) was accomplished in some of the patients and normal controls. All subjects gave informed consent to participate in the present study.

Analytical methods

The 24-h urine samples were collected at 4°C for determinations of FN, albumin, protein, and cr. Urine samples were stored at −20°C and analyzed at least within seven days. Blood specimens were obtained from the cubital vein in the morning after overnight fasting and at least 1 h of supine rest for measurements of fasting blood sugar (FBS) and HbA1c.

U-FN was measured by enzyme immunoassay. Urine samples (10 μl) and Dulbecco’s phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, 0.1% CHAPS and 0.05% thimerosal sodium salt were added to the wells which had been fixed with a monoclonal antibody raised against FN (Ohtsuka Assay Laboratories, Co., Ltd., Tokushima, Japan), then incubated for 2 h at room temperature. After washing with PBS containing Tween-20 (0.05%, v/v), biotinized second antibodies against FN were added to each well, and the mixture was incubated for 2 h at room temperature. The wells were rinsed with PBS, after which avidinized horse radish peroxidase was added, and the mixture further incubated

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (years)</th>
<th>Duration of diabetes (years)</th>
<th>Retinopathy (simple/proliferative)</th>
<th>Neuropathy</th>
<th>HbA1c (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 15)</td>
<td>51.9 ± 4.7</td>
<td>9.0 ± 0.8</td>
<td>18/13</td>
<td>30</td>
<td>9.1 ± 0.3</td>
</tr>
<tr>
<td>Diabetics (n = 59)</td>
<td>58.0 ± 1.6</td>
<td>7.5 ± 1.3</td>
<td>6/1</td>
<td>7</td>
<td>8.9 ± 0.5</td>
</tr>
<tr>
<td>G-O (n = 20)</td>
<td>56.6 ± 3.0</td>
<td>7.5 ± 1.3</td>
<td>9/9</td>
<td>17</td>
<td>9.3 ± 0.4</td>
</tr>
<tr>
<td>G-I (n = 31)</td>
<td>58.7 ± 1.9</td>
<td>9.5 ± 1.2</td>
<td>3/3</td>
<td>6</td>
<td>8.5 ± 0.5</td>
</tr>
<tr>
<td>G-II (n = 8)</td>
<td>58.8 ± 4.6</td>
<td>10.8 ± 1.2</td>
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</tbody>
</table>

Values are mean ± SEM.

Neuropathy was assessed by the extent of loss of vibratory sensation in the feet.
for 1 h. In less than five minutes after addition of o-phenylene diamine and H2O2, the amount of FN was measured spectrophotometrically at the wave-length of 492 nm with an automatic device (Type MPR-A4, TOSOH Co., Ltd.) using a purified human FN as a standard. Under these assay conditions, the detectability of FN determination was 15 ng/ml with interassay variation of 9.7% (n = 8) and intra-assay variation of 8.3% (n = 8). The U-FN was expressed as µg/g cr/24 h.

The amount of urinary albumin was measured by radioimmunoassay (Shionoria Albumin, Shionogi & Co., Ltd.). FBS was assayed enzymatically using an autoanalyzer (Glucose Auto and Stat, GA-1122, Kyoto Daiichi Kagaku Co., Ltd.). HbA1c was determined using an autoanalyzer (HLC-723GHB, Toyo Soda Manufacturing Co., Ltd.) coupled with high-performance liquid chromatography. Serum and urinary cr were measured using an autoanalyzer (COVAS FAR A, Baxter Co., Ltd.).

Statistical analysis
The values are expressed as mean ± SEM. Statistical analysis of the results was performed by Student’s t-test (unpaired samples). A null hypothesis was rejected when p < 0.05.

Results
Urinary excretion of FN
The U-FN was significantly greater in diabetic patients, as a group, than in normal controls (348.1 ± 48.3 vs. 108.6 ± 22.7 µg/g cr/24 h, p < 0.01). Figure 1 shows the U-FN levels in the different groups of diabetic patients. The U-FN tended to increase according to the evolution of diabetic nephropathy as assessed by the presence or absence of microalbuminuria or overt proteinuria; in G-II, the value was 1080.5 ± 184.0 µg/g cr/24 h (range 216.1 - 1726.8), which was significantly higher than in G-O (188.1 ± 34.3 µg/g cr/24 h, range 19.4 - 582.4; p < 0.01) and G-I (262.4 ± 21.9 µg/g cr/24 h, range 101.9 - 591.9, p < 0.01). Mean U-FN was higher in G-I than in G-O, although the difference did not reach a statistically significant level (p < 0.08).

Correlation of U-FN with renal functions and other study variables
As shown in Fig. 2, a highly significant negative correlation existed between the amounts of U-FN and Ccr in all the patients (r = -0.642, p < 0.01), but not in normal controls (r = 0.167, p = NS). U-FN was greater in the group of patients with a PSP-excretion rate of <25% than in that with the rate of ≥25% (673.0 ± 139.2 vs. 247.6 ± 28.0 µg/g cr/24 h, p < 0.05) (Fig. 3). Similarly, as shown in Fig. 4, patients with Fishberg’s concentration test values <1.025 (as maximum specific gravity) were found to excrete greater amounts of U-FN, than those with values of ≥1.025 (457.0 ± 77.0 vs. 231.5 ± 40.9 µg/g cr/24 h, p < 0.05).

U-FN did not correlate with FBS (r = 0.238, n = 39), HbA1c (r = -0.012, n = 59), age (r = 0.202, n = 59), or duration of disease (r = 0.040, n = 59) in patients.

U-FN and other diabetic complications
Table 2 summarizes the U-FN levels in diabetic patients with and without retinopathy and/or neuropathy. Compared with patients not affected by either retinopathy or neuropathy, U-FN was significantly increased in the group with retinopathy alone (258.3 ± 41.1 µg/g cr/24 h, p < 0.05). Such an increase was evident in the group with neuropathy only (333.4 ± 61.0 µg/g cr/24 h, p < 0.01) and in the group with both retinopathy and neuropathy (261.1 ± 34.9 µg/g cr/24 h, p < 0.05). In diabetic patients without retinopathy and neuropathy, the U-FN in G-I was significantly higher than in G-O (222.5 ± 28.5 vs. 116.1 ± 22.6 µg/g cr/24 h, p < 0.01).

Discussion
The notable findings from the present study are that (1) U-FN of diabetic patients was greater than that of matched normal controls, (2) U-FN tended to be higher...
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Fig. 2. Relationship between urinary excretion of fibronectin (U-FN) and endogenous creatinine clearance (Ccr) in normal controls (○) and diabetic patients (●). A highly significant negative correlation existed between these two variables in diabetic patients ($r = -0.642$, $p < 0.01$), but not in normal controls ($r = 0.167$, $p = \text{NS}$).

Fig. 3. Urinary excretion of fibronectin (U-FN) in diabetic patients when their renal function was assessed by the excretion rate of phenolsulfonphthalein (15 min after injection). Open triangles and closed circles show the results in patients with overt proteinuria and those without overt proteinuria, respectively.

in patients with diabetic nephropathy as assessed by the presence of either microalbuminuria or overt proteinuria, (3) in patients without retinopathy and neuropathy, U-FN was significantly higher in the group with microalbuminuria than in the group without it, (4) U-FN correlated negatively with Ccr in patients, but not in normal controls, and (5) patients with diabetic retinopathy and/or neuropathy also showed a greater amount of U-FN than did patients without such diabetic complications.

Although the observations made in this study are consistent with the findings of an earlier report in which U-FN was shown to increase in diabetic patients, the exact origin of FN in urine remains obscure. Since FN is synthesized by a variety of cells, including hepatocytes, fibroblasts, and vascular endothelial cells [3, 22, 23], and since several investigators have reported an increased level of plasma FN in diabetics, especially those with diabetic microangiopathy [8–10], it can be hypothesized that U-FN is, to some extent, of plasma origin and spills over from the circulation. However, studies in recent years have provided evidence that FN is present in quantities in kidney tissues. Further, FN has been demonstrated immunohistochemically in not only glomerular basement membranes, but also mesangial matrices (or cells) [17–19]. Biochemical characterization made it possible to distinguish U-FN and plasma FN in terms of their molecular size [20]. More importantly, messenger RNA (mRNA) of FN has been identified in the kidney cortex [24–26]. Taken collectively, these findings suggest that U-FN originates from kidney tissue.

Pettersson and Colvin [17], in 1978, first demonstrated that in the kidney specimens from human diabetes, immunopositive FN was increased in the mesangial area, along with an increment in the mesangial matrix. Later, Weiss and his colleagues [18] provided visual evidence that, in the human diabetic kidney, either the mesangium or glomerular capillary wall contained a sizable amount of immunoreactive FN. From the biological point of view that FN interacts with cell surfaces and with other FN molecules to influence cell growth, development, and cell-to-cell adhesion [3, 5–7], these findings suggest that FN plays a pathogenic role in the initiation and/or evolution of diabetic glomerular lesions characterized largely by thickened glomerular basement membranes and mesangium. Nonetheless, knowledge about the role of U-FN and mode of excretion of U-FN in diabetes is still far from adequate.
The present study clearly showed that U-FN is increased markedly in diabetics with overt proteinuria. In addition, U-FN in our patients with microalbuminuria showed a tendency to increase as compared with normal controls. Thus, it may be safely asserted that the increment in U-FN is relevant to the extent of glomerular injury affected by diabetes. Although the exact mechanism underlying the U-FN increase in patients with diabetic nephropathy remains speculative, recent advances in molecular biology have revealed that, in streptozotocin-induced rats, fibronectin mRNA levels are increased in the kidney cortex [24], suggesting a contributory role of hyperglycemia in the overproduction of fibronectin in kidney tissues. Thus, given that a large part of U-FN derives from the kidney, our study may provide clinical evidence that glycemic derangement is an important factor that increases fibronectin synthesis in the kidney, ultimately leading to diabetic glomerular injury. However, in the current study there was no significant relationship found between glycemic control, as judged from FBS and HbA1c values, and amounts of U-FN in diabetics, a finding not supportive of a hyperglycemic milieu as a pathogenic factor responsible for U-FN increase. We do not have a convincing explanation for this discrepancy, but accumulating evidence suggests that hyperglycemia per se can induce enhancement of fibronectin synthesis as well as overexpression of fibronectin mRNA, although neither is readily reversible even after the glucose level starts to normalize. Thus, further study is needed to clarify this important question by evaluating longitudinally the effect of glycemic control on the excretion of U-FN in diabetic patients.

Of interest in the present study is that U-FN correlated negatively with Ccr in diabetic patients, but not in normal controls. In addition, U-FN was found to increase in

<table>
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<tr>
<th>Subjects</th>
<th>U-FN (µg/g cr/24h)</th>
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<tbody>
<tr>
<td>Controls</td>
<td>108.6 ± 22.7</td>
</tr>
<tr>
<td>Diabetics (G-0, G-I)</td>
<td>233.3 ± 19.6*</td>
</tr>
</tbody>
</table>

Table 2. Urinary excretion of fibronectin (U-FN) in normal controls and diabetics (G-0, G-I) with and without diabetic retinopathy and neuropathy.

Values are mean ± SEM.
* p < 0.01 vs. normal controls.
** p < 0.05 vs. without retinopathy and neuropathy.
† p < 0.01 vs. G-0 without retinopathy and neuropathy.
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subjects who had reduced values of PSP excretion rate and Fishberg’s concentration test. Evolution of diabetic nephropathy is known to involve glomerular and tubular alterations both histologically and functionally. Thus, although we did not address the precise relationship between renal hemodynamic changes and U-FN excretion in the present study, these findings appear to support the concept that the rate of U-FN excretion increases as a consequence of progressive impairment of the kidney tissues affected by diabetes.

Finally, to our best knowledge, this study demonstrated for the first time that the amount of U-FN was higher in patients with diabetic neuropathy and/or retinopathy than in those without such complications. The precise clinical implication of this finding remains to be clarified. Microvascular disease, including retinopathy and nephropathy, occurs as a diabetic sequela. On the basis of the notion that vascular endothelial injury, which involves either renal microvasculature or other systemic microvessels, can cause enhancement of FN production, the increment in U-FN in diabetics with retinopathy observed in this study implies that the increase is a clinical feature of the diabetic microvascular lesions that also occur in renal microvessels.

In summary, the present study demonstrated that U-FN was increased in diabetic patients, as a group. Patients with diabetic nephropathy, characterized by the presence of overt proteinuria or microalbuminuria, were revealed to excrete a high amount of U-FN when compared to those without it. Furthermore, in diabetic patients, U-FN correlated negatively with Ccr. U-FN was also demonstrated to be elevated in patients with diabetic retinopathy and/or neuropathy. These findings not only suggest that U-FN could be a marker for diabetic nephropathy, but also imply that determination of U-FN will provide a better understanding of the clinical aspects of diabetic complications besides nephropathy.

Acknowledgement

The author wishes to thank Professor S. Fukuchi for his encouragement during this study, and Dr. K. Mizuno (Department of Internal Medicine, Odaka Municipal Hospital, Fukushima) for his invaluable comments. The technical assistance of Miss A. Hashimoto is greatly appreciated.

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