Galectin-3-positive cell infiltration in human diabetic nephropathy

Yuichi Kikuchi¹, Shuzo Kobayashi², Noriaki Hemmi¹, Ryota Ikee¹, Naomi Hyodo¹, Takamitsu Saigusa¹, Tamehachi Namikoshi¹, Muneharu Yamada¹, Shigenobu Suzuki¹ and Soichiro Miura¹

¹Second Department of Internal Medicine, National Defense Medical College, Tokorozawa, Saitama and
²Department of Nephrology and Kidney and Dialysis Center, Shonan Kamakura General Hospital, Kamakura, Kanagawa, Japan

Abstract

Background. Galectin-3 has several functions, such as cell proliferation, regulation of apoptosis and interaction of cell adhesion, and has a high binding affinity for advanced glycation end products. In animal models with diabetic nephropathy (DMN) or acute renal failure, galectin-3 is known to be upregulated. However, galectin-3 expression has not been investigated in human kidney diseases.

Methods. Using immunohistochemistry we examined galectin-3 expression in renal biopsy specimens obtained from 37 patients with nephropathy: DMN (n = 9), IgA nephropathy (n = 9), crescentic glomerulonephritis (n = 8), membranous nephropathy (n = 6) and minimal change nephrotic syndrome (n = 5).

Results. In normal human kidney, galectin-3 was found in distal tubuli, but not in glomeruli. However, galectin-3-positive cell infiltration was observed in glomeruli of 12 patients. Galectin-3-positive cells, also stained with CD68, were significantly more numerous in glomeruli of DMN than in glomeruli of other nephropathies. The ratio of galectin-3-positive cells to the total number of macrophages in tubules was also significantly increased in DMN. There was a significant correlation between the number of galectin-3-positive cells in glomeruli and urinary protein excretion in all patients (r = 0.616, P < 0.001). In diabetic patients, the number of galectin-3-positive cells in glomeruli closely correlated with the regression rate of renal function (r = −0.930, P < 0.005).

Conclusion. These findings suggest that galectin-3-positive cell infiltration may play an important role in the progression of DMN, and the degree of its expression may be predictive of poor prognosis of DMN.

Keywords: advanced glycation end product (AGE); diabetic nephropathy; foam cell; galectin-3; macrophage

Introduction

Galectin-3, originally described as a cell surface antigen for activated macrophages [1], has a C-terminal carbohydrate recognition domain and a domain consisting of proline- and glycine-rich repeats. Galectin-3 is detected in several inflammatory cells including mast cells, monocytes or macrophages, neutrophils and eosinophils [1–3]; it is also expressed by epithelial cells in a variety of organs [2,4]. It is found on the cell surface and within the extracellular matrix, as well as in the cytoplasm and nucleus. Galectin-3 has several biological roles including cell proliferation [5], differentiation and apoptosis [6]. On the cell surface, galectin-3 mediates cell to cell adhesion and cell to matrix interaction by binding to its complementary glycoconjugates such as laminin and fibronectin [7,8]. In addition, galectin-3 has also a high binding affinity for advanced glycation end products (AGE) and is considered to be a receptor of AGE [9,10].

In a normal rat kidney, galectin-3 is expressed in epithelial cells in distal tubules [11,12]. In a rat model of acute mesangial proliferative glomerulonephritis (by injection of anti-Thy1.1 antibody) [11], galectin-3 expression in distal tubules was increased, and it was found on glomerular macrophages in the acute phase. Later, galectin-3 was detected also in the mesangium and proximal tubules. We have already shown that in an ischaemia/reperfusion renal failure model in rats, galectin-3 expression increased in proximal and distal tubuli at 2–48 h after reperfusion and was followed by a normalization of the increased expression [12]. Therefore, those findings were considered to be acute reactions. On the other hand, in the study of
Galectin-3 in diabetic nephropathy

Subjects and methods

Patients

Percutaneous needle biopsy specimens were obtained from the kidneys of 37 patients. Of those, nine patients had DMN, nine, IgA nephropathy (IgAN), eight, crescentic glomerulonephritis (Cres GN), six, membranous nephropathy (MN) and five, minimal change nephrotic syndrome (MCNS). Four renal samples obtained from the healthy poles of nephrectomy specimens from patients with renal cell carcinoma were also examined. Renal biopsies on diabetic patients were performed to search for other renal diseases. All diabetics had type 2 diabetes mellitus. The diagnoses were based on clinical symptoms, laboratory data and histological findings. The laboratory findings on all patients are summarized in Table 1. Histological analyses were performed by staining with haematoxylin–eosin, periodic acid-Schiff (PAS), periodic acid-methenamine and Masson trichrome stains and by immunofluorescence against human IgG, IgA, IgM, C3, C4 and fibrinogen. This study was performed with informed consent from each patient.

Immunohistochemistry

We used monoclonal antibody against human gaelctin-3 (Affinity Bioreagent, Golden, CO, USA). Monoclonal antibodies against CD68 (PG-M1; DAKO, Carpinteria, CA, USA) to detect macrophages and HLA-DR (DAKO) to identify activated macrophages were also used on serial sections.

We used the kidney specimens (fixed in 10% formalin and paraffin embedded) obtained from the 37 patients for immunohistochemical staining. The staining was performed using the labeled streptavidin biotin (LSAB) method. The endogenous peroxidase activity in deparaffinized sections was blocked by incubation with 3% hydrogen peroxide for 10 min. After further blocking with 10% non-immune serum in phosphate-buffered saline (PBS) for 10 min, the sections were incubated with a primary antibody for human gaelctin-3 (1:200) overnight at 4°C in a high-humidity chamber. The incubation with primary antibodies to CD68 (1:100) and HLA-DR (1:100) was performed for 45 min at 37°C. These staining procedures required treatment with 0.04% proteinase K for 7 min for optimal antigen retrieval. Next, the sections were incubated with a DAKO LSAB system link-biotinylated secondary antibody (DAKO) for 10 min, followed by incubation with peroxidase-conjugated streptavidin at room temperature for 10 min. After washing with PBS, the sections were stained with a 3,3’-diaminobenzidine solution and then counterstained with haematoxylin. In western blots, the anti-gaelctin-3 antibody detected a band of ~30 kDa from cells transfected with recombinant human gaelctin-3. Negative controls consisted of either non-immune rabbit serum or omission of the primary antibodies.

Semi-quantification and statistical analysis

The number of gaelctin-3- or CD68-positive cells in each glomerulus was counted and averaged for each biopsy specimen. In each group, 85–262 (170.8±33.2) glomeruli were inspected. Global glomerulosclerosis and crescent formation in glomeruli were assessed in specimens stained with PAS, and the frequency of these glomeruli was presented as a percentage of the number of all glomeruli. The number of gaelctin-3- or CD68-positive cells in tubular lumina was also counted. The total number of these cells in the tubules of the

<table>
<thead>
<tr>
<th>Table 1. Clinical data of 37 patients</th>
</tr>
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<tbody>
<tr>
<td>DMN</td>
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<tr>
<td>-----</td>
</tr>
<tr>
<td>Number</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>SystBP (mmHg)</td>
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<tr>
<td>DiaBP (mmHg)</td>
</tr>
<tr>
<td>UP (g/day)</td>
</tr>
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<td>GFR (ml/min)</td>
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<td>BUN (mmol/l)</td>
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<tr>
<td>Cr (mmol/l)</td>
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<tr>
<td>TP (g/l)</td>
</tr>
<tr>
<td>Alb (g/l)</td>
</tr>
<tr>
<td>Tchol (mmol/l)</td>
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<tr>
<td>TG (mmol/l)</td>
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</tbody>
</table>

Values are represented as mean±SEM. BMI, body mass index; SystBP, systolic blood pressure; DiaBP, diastolic blood pressure; UP, urinary protein; BUN, blood urea nitrogen; Cr, creatinine; TP, total protein; Alb, albumin; Tchol, total cholesterol; TG, triglycerides.
renal cortex was divided by the total number of tubules in the renal cortex of each specimen and expressed as the number of positive cells per 100 tubules. The degree of interstitial fibrosis was scored semi-quantitatively in the Masson-stained specimens as follows: 0, no fibrosis; 1, mild (the range of fibrosis is \( \sim 25\% \)); 2, moderate (25\% - 50\%); 3, severe (50\% - ). These microscopic evaluations were performed by histologists without prior prejudicial information.

Results are expressed as mean ± SEM. Statistical analyses were performed using the Student t-test or analysis of variance (ANOVA) followed by Fisher’s PLSD test as appropriate. Correlation between galectin-3 expression and clinical data was assessed by Spearman’s correlation test. A \( P \)-value of \(< 0.05 \) was considered significant.

**Results**

In normal kidneys, galectin-3 expression was detected in epithelial cells of distal tubules, but not in glomeruli, proximal tubules or interstitium. Galectin-3 expression was detected in the distal tubules of all patients, but not, on the other hand, galectin-3 expression in their proximal tubules (Figure 1A). All interstitial foam cells (Figure 1B) and some cells in tubular lumina also showed galectin-3 positivity (Figure 1C).

Galectin-3-positive cell infiltration in glomeruli was observed in 12 of 37 patients (DMN, 6/9; IgAN, 3/9; Cres GN, 2/8; MN, 1/6; MCNS, 0/5), and the ratio of glomeruli with galectin-3-positive cells to all glomeruli in each groups was as follows: DMN, 22.5\% (± 8.2); IgAN, 4.3\% (± 2.5); Cres GN, 7.3\% (± 5.8); MN, 2.8\% (± 2.8); MCNS, 0\%. That ratio in patients with DMN was significantly greater than in patients with any of other diseases (\( P < 0.05 \)). A positive immunoreactivity in DMN is shown in Figure 1D and E. A study using serial sections showed that galectin-3-positive cells in glomeruli are also found to be CD68-positive (Figure 1F), most of which showed a positive immunostaining for HLA-DR. The numbers of macrophages (CD68-positive cells) in glomeruli in patients with DMN, IgAN and Cres GN were significantly increased compared with patients with MN and MCNS. Moreover, the numbers of macrophages in glomeruli in patients with IgAN and Cres GN were significantly increased compared with that in DMN (Figure 2A). In contrast, the number of galectin-3-positive cells in the glomeruli of patients with DMN was significantly higher than those in patients with the other diseases (Figure 2B). The proportion of glomerular galectin-3-positive cells vs CD68-positive cells in each group was as follows: DMN, 46.0\%; IgAN, 3.7\%; Cres GN, 4.0\%; MN, 4.4\%; MCNS, 0\% (\( P < 0.001 \)).

Regarding the number of galectin-3-positive cells in tubules of the renal cortex, there was no significant difference between the diseased groups (Figure 3A). However, the ratio of galectin-3-positive cells to the total number of macrophages in tubular lumina was, in diabetes, significantly higher compared with the other diseases (Figure 3B). In the interstitium, most of the galectin-3-positive cells were focally infiltrated foam cells. Nine patients (DMN, 4/9; IgAN, 3/9; Cres GN, 1/8; MN, 1/6; MCNS, 0/5) had foam cell infiltrations in the interstitium, and eight of them showed positive immunoreactivity for galectin-3 in glomeruli.

The clinical data and pathological findings in patients with (Group A) and without (Group B)

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**Fig. 1.** Immunohistochemistry of galectin-3 and CD68 in renal specimens. (A) Immunostaining of galectin-3 in a patient with MCNS. (B) Immunostaining of galectin-3 in a patient with MN with galectin-3-positive cell infiltration in glomeruli. All interstitial foam cells were galectin-3-positive. (C) Immunostaining of galectin-3 in a patient with Cres GN with galectin-3-positive cell infiltration in glomeruli. Some cells in tubular lumina were also galectin-3-positive. (D and E) Immunostaining of galectin-3 in patients with DMN. (F) Immunostaining of CD68 on serial section with (E).

**Fig. 2. (A)** The number of CD68-positive cells in glomeruli in five groups. * \( P < 0.005 \) vs MN and MCNS, ** \( P < 0.05 \) vs DMN. (B) The number of galectin-3-positive cells in glomeruli in five groups. ND, not detected. # \( P < 0.001 \) vs DMN.
Galectin-3-positive cell infiltration in glomeruli are summarized in Table 2. Although there was no significant difference between the two groups in urinary protein and glomerular filtration rate (GFR), urinary protein in Group A was higher and GFR in Group A was lower than those in Group B. In addition, 24 h urinary protein excretion was significantly correlated with the number of galectin-3-positive cells in all patients \( (r = 0.616, P < 0.001) \) (Figure 4).

Pathologically (Table 2), the frequency of global glomerulosclerosis and crescent formation in glomeruli tended to be greater in Group A than in Group B, though the difference did not reach statistical significance. Interstitial fibrosis in Group A was significantly more severe than in Group B \( (2.17 \pm 0.24 \text{ vs } 1.40 \pm 0.23, P < 0.05) \). No patients had been treated with angiotensin-converting enzyme inhibitors (ACE-I) in either group. Three patients (25%) in Group A and four (16%) in Group B had treatment with angiotensin II receptor blockers (ARB). However, treatment with ARB did not affect galectin-3 expression in this study. Regarding treatments with other agents, there were no significant differences between Groups A and B.

Table 3 summarizes the clinical data on the renal biopsies and pathological findings in nine patients with DMN, in whom galectin-3-positive cell infiltration was definitely detected. Urinary protein excretion was significantly correlated with the number of galectin-3-positive cells in glomeruli in diabetic patients \( (r = 0.688, P < 0.05) \), but there were no correlations between the number of galectin-3-positive cell infiltration in glomeruli and their other clinical characteristics, including GFR, glycated haemoglobin and so on. However, it is important to note that there is a significant correlation between the number of galectin-3-positive cells in glomeruli and the degree of decreased renal function expressed as \( \Delta \text{I/serum creatinine} \) \( (r = -0.930, P < 0.005) \) in seven diabetic patients (Figure 5). The follow-up data of two patients unfortunately were lost.

**Discussion**

In this study, we demonstrated that the number of galectin-3-positive cells in diabetic glomeruli significantly increased compared with other glomerular diseases. Because most galectin-3-positive cells in all glomerular disorders were also stained with CD68 and HLA-DR, these cells are considered to be activated macrophages. It has been suggested that infiltrated macrophages in kidneys might play an important role in the progression of IgAN, Cres GN and other renal diseases.
diseases [14,15]. Moreover, the glomerular infiltration of macrophages in diabetic kidneys is thought to also play a central role in the progression of nephropathy [16]. Furuta et al. [17] reported that the majority of glomerular infiltrated cells were macrophages and the number of them increased significantly in the moderate stage of glomerulosclerosis compared with the mild or advanced stages in diabetic patients. We found that the number of macrophages in the glomeruli of IgAN, Cres GN and DMN significantly increased compared with MN or MCNS. However, galectin-3-positive cell infiltrations in glomeruli are found to be mainly characteristic of diabetic kidneys, as shown in Figure 2B. Despite the prominent infiltration of glomerular macrophages (CD68-positive cells), the reason the number of galectin-3-positive cells was low in the glomeruli of IgAN or Cres GN remains unknown. There is a possibility, however, that the increased number of galectin-3-positive macrophages in the glomeruli of human diabetics might be specifically induced by diabetes-related substances, such as AGE. AGE in glomeruli may induce decreased renal function. In this regard, the correlation between the number of glomerular cells positive for galectin-3, which is a receptor of AGE, and Δ1/serum creatinine in diabetic patients is notable. Although such agents as ACE-I and ARB might affect galectin-3 expression or macrophage infiltration in glomeruli, there were no differences between Groups A and B in the use of such agents.

Galectin-3 has multiple functions including cell proliferation, cell differentiation, regulation of apoptosis, cell to cell and cell to matrix adhesion [5–8]. Moreover, it has a high affinity to AGE [9,10]. Indeed, increased expression of galectin-3 was observed in kidneys of streptozotocin-induced diabetic rats [13]. In galectin-3 knockout mice [18], the induction of diabetes significantly accelerated the progression of diabetic glomerulopathy compared with wild-type mice. Accordingly, there is a possibility that galectin-3 could be closely related to AGE-induced glomerular injury and the progression of DMN, although we cannot show a correlation between galectin-3 expression and the level of glycated haemoglobin or the duration of diabetes. On the examination of three patients with both IgAN and diabetes (duration, 5–16 years) without nephropathy, galectin-3-positive cells were not observed in glomeruli. This finding suggests that galectin-3 expression in glomeruli might be related to DMN but not to diabetes per se.

In tubular lesions, there was no significant difference in the number of galectin-3-positive cells between the diseased groups. However, many CD68-positive cells have been found in tubules in Cres GN and IgAN, as previously reported [15]. Therefore, the ratio of galectin-3-positive cells to the total of macrophages in tubular lumina in DMN was significantly higher than that in the other diseases. This finding also suggests that galectin-3 might play an important role in the progression of diabetic renal injury.

Interstitial foam cells also showed positive staining for galectin-3. The degree of interstitial fibrosis in patients with interstitial foam cell infiltration tended to be more severe than in those without, though it did not

Table 3. Clinical data, pathological findings and number of galectin-3 positive cells in diabetic patients

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age (years)</th>
<th>Duration of DM (years)</th>
<th>HbA1c (%)</th>
<th>Urinary protein (g/day)</th>
<th>GFR (ml/min)</th>
<th>Δ1/sCr (year (\times 10^{-3}))</th>
<th>Global sclerosis (%)</th>
<th>T-I change (score)</th>
<th>The number of Gal-3-positive cells (glomeruli)</th>
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<tbody>
<tr>
<td>1</td>
<td>48</td>
<td>10</td>
<td>8.8</td>
<td>12.7</td>
<td>45.0</td>
<td>-5.66</td>
<td>25.6</td>
<td>3</td>
<td>4.80 ± 1.07</td>
</tr>
<tr>
<td>2</td>
<td>45</td>
<td>15</td>
<td>9.5</td>
<td>6.4</td>
<td>42.5</td>
<td>-7.35</td>
<td>22.2</td>
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<td>3.60 ± 1.40</td>
</tr>
<tr>
<td>3</td>
<td>70</td>
<td>20</td>
<td>5.2</td>
<td>0.9</td>
<td>25.5</td>
<td>NT</td>
<td>46.7</td>
<td>2</td>
<td>2.46 ± 1.08</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>12</td>
<td>6.6</td>
<td>5.9</td>
<td>26.7</td>
<td>-2.81</td>
<td>27.8</td>
<td>2</td>
<td>1.80 ± 0.74</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
<td>4</td>
<td>6.2</td>
<td>1.6</td>
<td>30.2</td>
<td>NT</td>
<td>42.3</td>
<td>3</td>
<td>1.05 ± 0.35</td>
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<tr>
<td>6</td>
<td>29</td>
<td>7</td>
<td>7.4</td>
<td>3.5</td>
<td>40.8</td>
<td>-1.43</td>
<td>60.9</td>
<td>3</td>
<td>0.50 ± 0.27</td>
</tr>
<tr>
<td>7</td>
<td>58</td>
<td>9</td>
<td>6.5</td>
<td>0.7</td>
<td>24.4</td>
<td>0</td>
<td>50.0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>8</td>
<td>50</td>
<td>10</td>
<td>7.0</td>
<td>6.8</td>
<td>47.7</td>
<td>-0.32</td>
<td>7.7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>60</td>
<td>25</td>
<td>6.5</td>
<td>0.4</td>
<td>74.1</td>
<td>-1.12</td>
<td>8.3</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Hb, haemoglobin; sCr, serum creatinine; T-I, tubulo-interstitial; Gal-3, galectin-3; NT, not tested. Δ1/sCr is calculated by \((1/sCr at 1 year after) – (1/sCr at renal biopsy)\).
reach a statistical significance (2.00 ± 0.29 vs 1.54 ± 0.22). Interestingly, 67% (8/12) of patients with galectin-3-positive cell infiltration in glomeruli had interstitial foam cell infiltration, while of 25 patients without galectin-3-positive cell infiltration in glomeruli only one had interstitial foam cells. The foam cell formation is known to be mediated by dyslipidaemia. In an investigation of apolipoprotein-E deficient mice that showed remarkably high cholesterolaeemia, Mac-2 (galectin-3)-positive cell infiltration was observed in glomeruli [19]. Although serum total cholesterol and triglycerides were higher in patients with MCNS and MN than in those with other nephropathies, these serum levels did not differ significantly between patients with and without galectin-3-positive cell infiltration. However, since renal biopsies in most of the patients with MCNS and MN were performed after the disease crisis, the hyperlipidaemia found in these patients could have been transitory. Persistent dyslipidaemia, which has been observed in a majority of diabetic patients, might also induce galectin-3 upregulation.

Our study does not clearly demonstrate if galectin-3 expression in patients with DMN could play a role in protecting from or accelerating the progression of this disease. Along with macrophage infiltration in diabetic kidneys [17], galectin-3 expression in glomeruli was also prominent in the histologically moderate stage of DMN (at a global glomerulosclerosis ratio of 10–50%) (Table 3). We believe that the predominant immunoreactivity to galectin-3 in a moderate stage of diabetic kidney is an important finding in terms of the various roles of macrophage infiltration, since activated macrophages are known to produce a variety of cytokines and growth factors. Galectin-3-positive macrophages might also produce inflammatory mediators and promote additional macrophage infiltration in diabetic kidneys. In this regard, it was recently reported that in experimentally induced diabetic rats, the anti-inflammatory therapy by mycophenolate mofetil suppressed macrophage infiltration in the glomeruli and protected them from the progression of nephropathy [20]. One particular finding to note is that there was a strongly significant correlation between the number of galectin-3-positive cells in diabetic glomeruli and the decline of renal function shown as Δ1/serum creatinine per year in our study. Anti-inflammatory therapy in the moderate stage of DMN may suppress the progression of this disease.

In conclusion, galectin-3-positive cell infiltration in glomeruli in patients with DMN is significantly higher compared with those with other renal diseases. Galectin-3 expression might be induced by AGE formation or persistent dyslipidaemia, and it might be related to the progression of DMN.

Conflict of interest statement. None declared.

References

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