Redistribution of connexin43 expression in glomerular podocytes predicts poor renal prognosis in patients with type 2 diabetes and overt nephropathy

Kazutomo Sawai¹, Masashi Mukoyama¹, Kiyoshi Mori¹, Hideki Yokoi¹, Masao Koshikawa¹, Tetsuro Yoshioka¹, Ryuji Takeda², Akira Sugawara¹, Takashi Kuwahara³, Moin A. Saleem⁴, Osamu Ogawa⁵ and Kazuwa Nakao¹

¹Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto, ²Department of Life Science and Chemistry, Kinki University Graduate School of Agriculture, Nara, ³Department of Nephrology, Osaka Saiseikai Nakatsu Hospital, Osaka, Japan, ⁴Children’s Renal Unit, University of Bristol, Bristol, UK and ⁵Department of Urology, Kyoto University Graduate School of Medicine, Kyoto, Japan

Abstract

Background. Significance of podocyte injury in the progression of diabetic nephropathy is not well-understood. In this study, we examined whether alteration of gap junction protein connexin43 (Cx43) expression in podocytes is associated with the progression of overt diabetic nephropathy.

Methods. We recruited 29 type 2 diabetic patients with overt nephropathy who underwent renal biopsy. Nephrectomized kidney samples obtained from seven subjects with localized neoplasm and biopsy specimens from five patients diagnosed as minor glomerular abnormalities were used as controls. Cx43 staining on paraffin-embedded kidney sections were studied by immunohistochemistry.

Results. In controls, Cx43 was expressed at podocytes in a linear pattern along the glomerular basement membrane. In contrast, downregulation and loss of uniformly linear staining of Cx43 (Cx43 heterogeneity) in podocytes were observed in diabetic nephropathy. Cx43 intensity correlated with current renal function ($R = 0.647, P < 0.005$), whereas the magnitude of Cx43 heterogeneity correlated well with the degree of future decline in renal function ($R = -0.705, P < 0.001$).

Conclusions. Alteration of Cx43 expression in podocytes was closely associated with the progression of overt diabetic nephropathy. These results indicate that change in Cx43 expression at podocytes represents a progressive stage in overt diabetic nephropathy and that it may be a convenient way to predict future decline in renal function.

Keywords: connexin43; glomerular visceral epithelial cell; overt diabetic nephropathy; pathogenesis; podocyte; prognosis

Introduction

Podocytes are highly differentiated glomerular cells crucially involved in establishing selective permeability properties of the glomerular filtration barrier. Injury to podocytes leads to proteinuria, a hallmark of most glomerular diseases [1]. Decline in renal function in renal diseases is due to the progressive loss of viable nephrons. In majority of cases, the loss of nephrons most likely starts with the injury of podocytes [1]. Recent studies have revealed that podocyte loss or decrease in podocyte density is an early event in diabetic nephropathy [2], and that podocyte number predicts long-term urinary albumin excretion [3]. Podocyte loss in animal models of diabetic nephropathy is also reported [4]. These results suggest that podocyte injury is implicated in the pathogenesis of diabetic nephropathy, but the role of podocytes in the progression of diabetic nephropathy is not well-understood.

The rate of decline in glomerular function in overt diabetic nephropathy is highly variable from individual to individual with type 1 and type 2 diabetes [5]. Early identification of patients with progressive diabetic nephropathy is of great value, since such patients are in need for an intensified therapeutic regimen. A growing body of information supports the view that risk factors for diabetic nephropathy may be intrinsic to the kidney [5]. This notion is supported by the fact that there is a marked variability in the rate of development of kidney lesions of diabetic nephropathy.
Cx43 expression in human diabetic nephropathy

in transplanted kidneys, despite all the recipients having renal failure secondary to diabetic nephropathy [6]. However, specific ‘markers’ within the kidney for predicting future decline in renal function remain to be established.

Connexins are integral membrane proteins that form hexamers called connexons. Two connexons located in the plasma membrane of adjacent cells form gap junctions, which are intercellular channels that permit the passage of small molecules such as small metabolites, ions and second messengers [7]. At least 20 rodent and 21 human connexins have been identified [7]. Among them, connexin43 (Cx43) is expressed most abundantly and by a variety of cell types [7]. In the kidney, nine connexins are known to be expressed: Cx26, Cx30.3, Cx31, Cx32, Cx37, Cx40, Cx43, Cx45 and Cx46 [8]. Previous studies have shown that Cx43 is expressed at podocytes in normal and diseased human kidney [9,10], but Cx43 expression in diabetic nephropathy and its significance remain totally unknown.

In this study, we examined the changes in glomerular Cx43 staining of type 2 diabetic patients with overt nephropathy, comparing them with normal controls. Furthermore, we investigated whether the magnitude of alteration in Cx43 expression at podocytes could be a prognostic marker for the progression of diabetic nephropathy.

**Subjects and methods**

**Subjects**

Needle renal biopsy tissues of 29 type 2 diabetic patients with overt nephropathy were obtained from those treated between 1999 and 2005 at Kyoto University Hospital or at Saiseikai Nakatsu Hospital. The patients underwent renal biopsy because of the presence of haematuria, and/or the absence of retinopathy, and/or a known history of diabetes. The presence of overt diabetic nephropathy was confirmed based on established criteria [5]. No patient received steroids or immunosuppressive drugs. The patients were followed up for 10–49 months (mean, 23 ± 3 months). For normal controls, tissues obtained from seven subjects using uninvolved portions of surgically removed kidneys afflicted with localized neoplasm, and five biopsy samples from patients with minor glomerular abnormalities were used. Histopathological examination of control tissues excluded any glomerular diseases. The samples were fixed in Dubosq-Brazil solution and embedded in paraffin. Table 1 summarizes the details of the analysed materials. This study was approved by the Human Research Committee of Kyoto University Graduate School of Medicine.

**Immunohistochemistry**

Immunohistochemical analysis for Cx43 and Wilms’ tumor-1 (WT1) was performed as previously described [4] with some modifications. In brief, deparaffinized 3 μm kidney sections were treated with autoclave heating (10 min in 10 mM citrate buffer, pH 6.0). After blocking of endogenous peroxidase with 1.5% H2O2 for 15 min at 22°C, sections were incubated with 1% Triton-X in phosphate-buffered saline (PBS) for 20 min at 22°C, washed three times with PBS for 5 min and incubated with 10% normal goat or donkey serum in PBS for 10 min. Rabbit antibodies against Cx43 (C-6219, Sigma-Aldrich, St. Louis, USA) or human WT1 (sc-192, Santa Cruz Biotechnology, Santa Cruz, USA) were diluted 1:50 in PBS containing 1% bovine serum albumin (1% BSA/PBS), and the mixture was incubated for 1 h at 22°C. For Cx43 staining, after incubation with streptavidin-horseradish peroxidase (HRP) (DakoCytomation, Kyoto, Japan) for 20 min at 22°C, the sections were incubated with anti-rabbit biotin (Vector, Burlingame, USA) for 30 min at 22°C. The sections were processed with 3,3-diaminobenzidine and counterstained with hematoxylin. For WT1 staining, after blocking of endogenous phosphatase by incubation with 2 mM levamisole for 5 min three times at 22°C, the sections were incubated with alkaline phosphatase-conjugated secondary antibodies (711-055-152, donkey anti-rabbit IgG, Jackson ImmunoResearch, West Grove, USA) diluted 1:100 in 1% BSA/PBS for 30 min at 22°C. The sections were processed with nitroblue tetrazolium-bromochloroindolylphosphate toluidine (1-697-471, Roche Diagnostics, Mannheim, Germany) in alkaline buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM MgCl2) and counterstained with Kernechtrot Stain Solution (Muto Pure Chemicals, Tokyo, Japan). Non-immune rabbit serum was used as negative controls.

**Evaluation of Cx43 staining**

Evaluation of Cx43 staining was classified by the intensity of Cx43 staining as scores 0–4 (0, no expression detectable;
1, only weak expression; 2, detectable expression; 3, high expression as observed in normal controls; 4, highest expression, higher than normal controls) and by the degree of Cx43 heterogeneity, defined as the loss of uniformly linear Cx43 staining pattern due to segmental thickening, thinning and loss of Cx43 staining, as scores 0–3 (0, without heterogeneity as in normal controls; 1, segmental heterogeneity, segmental heterogeneity with conservation of more than half of glomerular linear Cx43 staining; 2, moderate heterogeneity, heterogeneity with less than half of glomerular linear Cx43 staining; 3, global heterogeneity, global heterogeneity with almost no preservation of linear Cx43 staining; see Figures 1 and 2 for representative Cx43 staining). Examination of Cx43 staining intensity and Cx43 heterogeneity was done by two independent reviewers using normal controls and representative diabetic nephropathy samples as relative standards, and the scores were averaged. At least six glomeruli per biopsy were evaluated at high-power magnification, and the scores were averaged.

The number of podocytes per glomerulus cross section was determined by the mean number of WT1-positive cell number per glomerular cross section, excluding parietal epithelial cells.

**Cell culture and western blotting**

A conditionally immortalized human podocyte was cultured as described previously [11]. In brief, these cells proliferate when cultured at 33°C, whereas they halt growing and begin to differentiate to express podocyte-specific genes when cultured at 37°C. Podocytes were cultured with RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich) and insulin–transferrin–selenium media supplement (Sigma-Aldrich). Cells were differentiated at 37°C for 2 weeks without passage, and cultured with RPMI 1640 containing 10% FCS with 5.5 mM glucose (normal glucose) or 25 mM glucose (high glucose) for 8 days. Cells were used between passages 15 and 18.

Western blot analysis was performed as described [4]. After incubation with 50% trichloroacetic acid on ice for 10 min, cells were centrifuged at 15,000 x g. Precipitates were resuspended in sample buffer containing 2% 2-mercaptoethanol, 125 mM Tris, 20% glycerol, 0.001% bromophenol blue and 4% sodium dodecyl sulfate (SDS), and separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane (Bio-Rad, Hercules, USA). After incubation with rabbit antibodies against Cx43 (C-6219, Sigma-Aldrich) or mouse antibodies against β-actin (A-2547, Sigma-Aldrich), immunoblots were developed with HRP-conjugated anti-rabbit IgG (Amersham, Arlington Heights, USA) or HRP-conjugated anti-mouse antibody (Santa Cruz) and Lumiglo Reagent (Cell Signaling Technology, Danvers, USA).

**Statistics**

Data are expressed as means±SEM, unless otherwise indicated. Correlation coefficients were calculated by Spearman’s method, and between-group differences were determined by ANOVA with Kruskal–Wallis method.

---

**Fig. 1.** Cx43 staining in normal control and in diabetic nephropathy. Cx43 staining (A, B, E, F, I, J, stained with anti-Cx43 antibody, brown), WT1 staining (C, G, K, stained with anti-WT1 antibody, purple), and Periodic acid-Schiff (PAS) staining (D, H, L) in normal controls (A–D) and in diabetic patients (E–L) are shown. Podocytes are detected as nuclear WT1 positive cells outside the glomerular basement membrane (GBM) (C, G, K, white arrows). In normal controls, Cx43 staining in podocytes showed a linear to fine granular pattern along the GBM (A, B, black arrowheads). In case 1 (sCr 0.8 mg/dl at the time of biopsy and has a stable glomerular function after 3 years, E–H, Cx43 heterogeneity score, 1; Cx43 intensity score, 2), partial loss of Cx43 (F, white arrowhead) was present but the Cx43 staining pattern seems uniform. In contrast, in case 2 (sCr 0.8 mg/dl at the time of biopsy, but reached end-stage renal failure in 2 years, I–L, Cx43 heterogeneity score, 3; Cx43 intensity score, 2), segmental loss or thinning of Cx43 expression (I, J, white arrowheads) and segmental thickening of Cx43 expression (I, J, black arrows) were prominent. Scale bars, 50 μm (A, C–E, G–I, K, L) and 15 μm (B, F, J), sCr, serum creatinine.

**Fig. 2.** Representative figures for Cx43 heterogeneity and Cx43 intensity score. Representative figures for Cx43 heterogeneity score (Cx43 hetero score, A–D): 0, without heterogeneity as in normal controls (A); 1, segmental heterogeneity, heterogeneity with conservation of more than half of glomerular linear Cx43 staining (B); 2, moderate heterogeneity, heterogeneity with less than half of glomerular linear Cx43 staining (C); 3, global heterogeneity, global heterogeneity with almost no preservation of linear Cx43 staining (D). Representative figures for Cx43 intensity score (Cx43 intensity score, E–I): 0, no expression (E); 1, only weak expression (H); 2, detectable expression (G); 3, high expression as observed in normal controls (F); 4, highest expression, higher than normal controls (E). Western blot analysis of Cx43 protein levels in cultured human podocytes grown in normal or high-glucose medium (J, K). Cx43 protein levels are reduced in podocytes cultured in high-glucose medium (25 mM) by 20% compared with cells grown in normal medium (5.5 mM) n = 6.
A level of $P < 0.05$ was considered statistically significant. Statistical analysis was performed using StatView (R) software package (Abacus Concepts, Berkeley, USA) and JUSE-package software version 2.42 (Union of Japanese Scientist and Engineers, Tokyo, Japan).

**Results**

Cx43 staining in normal controls showed a uniformly linear to a fine granular pattern along the glomerular basement membrane (GBM), indicating that Cx43 was almost exclusively expressed at podocytes within the glomeruli (Figure 1A and B). In diabetic nephropathy, Cx43 staining was still predominant in podocytes, but Cx43 staining in podocytes was down-regulated compared with normal controls (Figure 1E, F, I and J). In some patients, loss of uniformly linear Cx43 pattern in podocytes, i.e. segmental loss or thinning of Cx43 staining and thickened Cx43 staining in cytoplasm of podocytes, was observed (Figure 1I and J). Such Cx43 redistribution in podocytes was evaluated as ‘Cx43 heterogeneity’ by scoring from 0 (normal) to 3 (most advanced) (Figure 2A–D).

Magnitude of Cx43 intensity as scored from 0 (no signal) to 4 (highest expression, higher than normal controls) (Figure 2E–I) had no significant correlation with the degree of Cx43 heterogeneity (Figure 3E, Table 2). Cx43 intensity score correlated positively with creatinine clearance (Figure 3A) and inversely with serum creatinine (Figure 3B) at the time of renal biopsy. Cx43 heterogeneity score also had a weak correlation with creatinine clearance (Table 2). Although Cx43 intensity score did not show a significant association with the change in renal function (Table 2), Cx43 heterogeneity score closely correlated with the future decline in renal function, expressed as a monthly decline of inverse serum creatinine ($\Delta 1/sCr$) (Figure 3C, Table 2). Change in renal function correlated inversely with some of the previously reported promoters of diabetic nephropathy, such as the level of urinary protein at renal biopsy, systolic blood pressure, serum creatinine, BUN and urinary protein at follow-up [5] (Table 2). Cx43 intensity correlated positively with podocyte number per glomerular cross section, and inversely with systolic blood pressure at renal biopsy (Figure 3D, Table 2).

To evaluate the mechanism of the altered Cx43 expression at podocytes in diabetic nephropathy, conditionally immortalized human podocyte cell line was cultured with a high-glucose (25 mM) or normal-glucose (5.5 mM) medium. High glucose decreased Cx43 expression by 20% compared with normal glucose (Figure 2J and K). These results show that, unlike murine podocytes, Cx43 is consistently and constitutively expressed at human podocytes, and that Cx43 expression can be decreased by high-glucose condition.

![Graph A](image1)

**Fig. 3.** Correlation of Cx43 intensity score and Cx43 heterogeneity score with renal function and proteinuria in patients with overt diabetic nephropathy. Cx43 intensity score significantly correlated positively with Ccr (A, $n = 21$, $R = 0.647$, $P < 0.005$), and inversely with serum creatinine (B, $n = 28$, $R = -0.638$, $P < 0.001$). Cx43 heterogeneity score correlated closely with the future decline in renal function (C, $n = 22$, $R = -0.705$, $P < 0.001$, observation period 23 ± 3 months), and positively with urinary protein at renal biopsy (D, $n = 27$, $R = 0.535$, $P < 0.01$). Cx43 intensity score did not significantly correlate with Cx43 heterogeneity score (E, $n = 28$). Ccr, creatinine clearance; sCr, serum creatinine; RB, renal biopsy.
Table 2. Spearman’s correlation coefficient for Cx43 intensity score, Cx43 heterogeneity score and change in renal function vs clinical parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cx43 heterogeneity score</th>
<th>Cx43 intensity score</th>
<th>Change in renal function (Δ1/sCr/month)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx43 heterogeneity score</td>
<td>1</td>
<td>-0.323</td>
<td>-0.705(*)</td>
</tr>
<tr>
<td>Cx43 intensity score</td>
<td>-0.323</td>
<td>1</td>
<td>0.253</td>
</tr>
<tr>
<td>Change in renal function (Δ1/sCr/month)</td>
<td>-0.705(*)</td>
<td>0.253</td>
<td>1</td>
</tr>
<tr>
<td>Age (years)</td>
<td>-0.095</td>
<td>-0.063</td>
<td>-0.025</td>
</tr>
<tr>
<td>sCr at renal biopsy (mg/dl)</td>
<td>0.459(*)</td>
<td>-0.638(*)</td>
<td>-0.255</td>
</tr>
<tr>
<td>BUN at renal biopsy (mg/dl)</td>
<td>0.306</td>
<td>-0.494(*)</td>
<td>0.023</td>
</tr>
<tr>
<td>HbA1c at renal biopsy (%)</td>
<td>-0.249</td>
<td>0.315</td>
<td>0.119</td>
</tr>
<tr>
<td>Ccr at renal biopsy (ml/min)</td>
<td>-0.402</td>
<td>0.647(*)</td>
<td>0.235</td>
</tr>
<tr>
<td>Urinary protein at renal biopsy (g/gCr)</td>
<td>0.535(*)</td>
<td>-0.002</td>
<td>-0.606(*)</td>
</tr>
<tr>
<td>sCr at follow-up (mg/dl)</td>
<td>0.604(*)</td>
<td>-0.438(*)</td>
<td>-0.525(*)</td>
</tr>
<tr>
<td>BUN at follow-up (mg/dl)</td>
<td>0.686(*)</td>
<td>-0.542(*)</td>
<td>-0.605(*)</td>
</tr>
<tr>
<td>HbA1c at follow-up (%)</td>
<td>-0.161</td>
<td>0.428</td>
<td>0.000</td>
</tr>
<tr>
<td>Ccr at follow-up (ml/min)</td>
<td>-0.625</td>
<td>-1.000</td>
<td>0.500</td>
</tr>
<tr>
<td>Urinary protein at follow-up (g/gCr)</td>
<td>0.467</td>
<td>0.08</td>
<td>-0.599(*)</td>
</tr>
<tr>
<td>Podocyte/glomerulus (cells/glomerulus)</td>
<td>-0.020</td>
<td>0.456(*)</td>
<td>0.171</td>
</tr>
<tr>
<td>SBP at renal biopsy (mmHg)</td>
<td>0.296</td>
<td>-0.424(*)</td>
<td>-0.042</td>
</tr>
<tr>
<td>DBP at renal biopsy (mmHg)</td>
<td>0.203</td>
<td>-0.252</td>
<td>0.045</td>
</tr>
<tr>
<td>mBP at renal biopsy (mmHg)</td>
<td>0.189</td>
<td>-0.358</td>
<td>0.081</td>
</tr>
<tr>
<td>SBP at follow-up (mmHg)</td>
<td>0.412</td>
<td>-0.098</td>
<td>-0.593(*)</td>
</tr>
<tr>
<td>DBP at follow-up (mmHg)</td>
<td>-0.263</td>
<td>-0.041</td>
<td>0.262</td>
</tr>
<tr>
<td>mBP at follow-up (mmHg)</td>
<td>0.118</td>
<td>-0.095</td>
<td>-0.298</td>
</tr>
</tbody>
</table>

\*P < 0.05, \(\*\) P < 0.01, \(\#\) P < 0.005, \(\#\) P < 0.001.

sCr, serum creatinine; BUN, blood urea nitrogen; Ccr, creatinine clearance; SBP, systolic blood pressure; DBP, diastolic blood pressure; mBP, mean blood pressure.
Recent reports show that some unapposed connexons, called hemichannels, connect the intracellular and extracellular space [7]. Cx43 heterogeneity may indicate Cx43 hemichannel activation in podocytes, which may further lead to podocyte injury, as is the case with renal proximal tubule cells in ischemic injury [17].

As we have shown in this study, persistent high glucose is a candidate mechanism for decrease in Cx43 intensity and the development of Cx43 heterogeneity in podocytes. High glucose is reported to reduce Cx43 expression in various other cell types [18]. Phosphorylation of Cx43, by kinases including protein kinase-C and mitogen-activated protein kinase [7], may also alter Cx43 expression and function, such as the regulation of trafficking, assembly, channel gating, internalization and degradation [7]. Although, there was no significant correlation between Cx43 heterogeneity and the level of glycaemic control in our study, Cx43 heterogeneity may result from the combined effects of factors aforementioned that were involved in the progression of diabetic nephropathy [19].

Mechanisms for Cx43 alteration in podocytes remain to be elucidated in the future.

Cx43 is abnormally expressed in myocardium but is also found in many other cell types [7]. Although Cx43 knockout mice seem to have normal kidney morphology [20], Cx43 expression in rodents and humans are quite different. In rodents, glomerular Cx43 expression is detected in the extraglomerular mesangium, mesangial cells and sparsely along the glomerular capillary wall in normal glomerulus [12]. In rodents, Cx43 becomes apparent only after podocyte injury [12]. In humans, however, Cx43 is most abundantly expressed in podocytes within the glomeruli [9,10]. Significance of Cx43 gene mutation in renal disease remains to be explored.

In summary, the findings of the present study indicate that alteration of podocyte Cx43 expression proceeds even after the establishment of overt diabetic nephropathy, being involved in further progression of the disease. Further analysis of the mechanism of Cx43 redistribution may elucidate the role of podocytes in the progression of overt diabetic nephropathy. Quantification of Cx43 staining may also be an easy and convenient way to assess podocyte damage and to predict future decline in renal function in diabetic patients with overt nephropathy.

Acknowledgements. The authors gratefully acknowledge Dr P.W. Mathiesson for providing human podocytes, Dr K. Nagahama and Dr H. Nishiyama for discussion, Ms. J. Nakamura and Ms. S. Suito for technical assistance, and Ms. A. Sonoda and Ms. S. Doi for secretarial assistance. This work was supported in part by research grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology, the Japanese Ministry of Health, Labour and Welfare, and Yamanouchi Foundation for Research on Metabolic Disorders.

Conflict of interest statement. None declared.

References


Received for publication: 18.8.05
Accepted in revised form: 18.4.06