Immunohistochemical Examination of Cyclooxygenase-2 and Renin in a KK-\(A^\gamma\) Mouse Model of Diabetic Nephropathy

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Abstract: The renin-angiotensin system plays a central role in the pathological mechanisms of diabetic nephropathy and is regulated by renal expression of cyclooxygenase-2 (COX-2). In the present study, the kidneys of diabetic KK-\(A^\gamma\) mice, a model of human type 2 diabetes, were investigated histologically and immunohistochemically at 8, 12, 16, and 20 weeks of age, and changes in renal lesions and expression of COX-2 and renin were evaluated quantitatively. Glomerular damage, characterized by expansion of mesangial matrices and nodular lesions, was observed in the kidneys of these mice. The glomerular sclerosis score gradually increased with age and was significantly higher than those of age-matched control C57BL/6 mice at 12, 16, and 20 weeks of age. Although mild tubulointerstitial damage was observed, there was no significant change in the interstitial fibrosis score. These findings were considered early diabetic nephropathy changes. COX-2-positive signals were consistently detected in the macula densa cells of the thick ascending limbs in all KK-\(A^\gamma\) mice, with a slightly higher score observed at 8 weeks of age. No COX-2-positive signals were detected in C57BL/6 mice. Renin-positive signals were commonly detected in the juxtaglomerular arterioles, and the scores in KK-\(A^\gamma\) mice increased at 16 weeks and decreased at 20 weeks of age. The present study demonstrated activation of renal COX-2 and renin expression in diabetic KK-\(A^\gamma\) mice at different stages. This finding suggests that these two enzymes contribute to the development and progression of diabetic nephropathy via different mechanisms.

Key words: cyclooxygenase-2, diabetic nephropathy, KK-\(A^\gamma\) mouse, renin, type 2 diabetes
successfully replicated only in a limited number of models, and animal models of spontaneous DN, especially type 2 diabetes, are few and valuable.

The KK-A'y/Ta mouse is a model of type 2 diabetes and was produced by transferring the yellow obese gene (A' allele) into the KK/Ta mouse [22]. The DN phenotypes in KK-A'y mice are more severe than those in the original KK mouse [23], and a recent study showed that DN in KK-A'y mice resembled human DN, both histopathologically and immunohistochemically [11].

An interaction between hemodynamic and metabolic factors induces DN [28]. Although the renin-angiotensin system (RAS) is a major physiological mechanism that regulates hemodynamics, including systemic blood pressure and renal glomerular capillary pressure, it also plays a central role in DN pathogenesis. Moreover, the beneficial effects of angiotensin-converting enzyme inhibitor (ACEi) and angiotensin receptor blocker (ARB) in preventing DN are widely accepted in current medical science [1].

Renin, a key enzyme of the RAS, is released from juxtaglomerular cells. In studies over the last decade, it has been demonstrated that the stimulation of renin release is regulated by renal cyclooxygenase (COX)-2 expression [3, 7, 8]. Many studies using streptozotocin (STZ)-induced type 1 diabetic rats have shown an increase in renal COX-2 production in DN [2, 16, 24], and similar findings have also been reported in type 2 diabetic rats [17, 31]. Although these findings in rats are thought to be clinically worthy, further research in other species is necessary for the development of novel clinical strategies for DN prevention, as many studies have shown that species-specific differences in RAS and renal COX-2 expression exist [9, 10]. Therefore, in the present study, the kidneys of KK-A'y mice, as a model of human type 2 diabetes, were investigated histologically and immunohistochemically, and changes in the renal expressions of renin and COX-2 during DN progression were quantitatively evaluated.

Materials and Methods

The present study was conducted in accordance with the Guidelines for Animal Experimentation of Kagoshima University, Japan (accession No. H19-agri-015). Animals

Male diabetic KK-A'y/TaJcl (n=20) and non-diabetic C57BL/6NJcl mice (n=20) were purchased from CLEA Japan (Tokyo, Japan). Glycosuria of each mouse was checked with diagnostic paper (HI-TESPER-G; Eiken Chemical, Tokyo, Japan). The mean (standard error [SE]) age of onset of glycosuria in KK-A'y mice was 41.5 (1.1) days old. All C57BL/6 mice showed no glycosuria. The mice were sacrificed by exsanguination under ketamine-medetomidine anesthesia at 8, 12, 16, and 20 weeks of age (n=5 per group per strain). The examination ages were determined according to a previous report on DN KK-A'y mice [11].

Tissue preparations

Kidneys were quickly removed and cut perpendicular to the long axis and fixed in 10% neutral-buffered formalin for 24 h at 4°C. After routine embedding in paraffin, 3-µm sections were cut and stained with periodic acid Schiff (PAS) and Masson’s trichrome. Immunohistochemistry was performed using peroxidase-conjugated streptavidin (DakoCytomation, Glostrup, Denmark). Anti-COX-2 polyclonal antibody (1:800 dilution; Cayman Chemical, Ann Arbor, MI, USA) and anti-renin polyclonal antibody (1:3,000 dilution; supplied by Dr. Murakami, University of Tsukuba, Japan) were used as primary antibodies. For the negative control sections, non-immunized rabbit immunoglobulin (IgG) (DakoCytomation) was used instead of primary antibody. The sections were incubated with primary antibodies overnight at 4°C for COX-2 and 60 min at room temperature for renin. Biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1:200 was used as the secondary antibody. Immunopositive signals were detected using a 3,3’-diaminobenzidine system (DAB Buffer tablet; MERCK, Darmstadt, Germany). The reactions were stopped by adding cold distilled water; then, the sections were counterstained using Mayer’s hematoxylin. For COX-2 detection, antigen retrieval treatment was performed after deparaffinization. The sections were microwaved in 10 mM citrate buffer (pH 6.0) and then treated with 0.3% Triton X-100 in 10 mM phosphate-buffered saline. No antigen retrieval treatment was required for renin detection. For the morphometric analysis, the sections prepared for immunohis-
Morphometric analysis

Randomized quantitative analysis was performed according to previously established methods [25, 31]. First, the extent of glomerulosclerosis was evaluated using a previously described semiquantitative scoring system. Briefly, we examined approximately 100 glomeruli per animal and graded the severity of the sclerotic lesions from 0 to +4, where 0 = normal, +1 = up to 25% sclerosis, +2 = 26–50% sclerosis, +3 = 51–75% sclerosis, and +4 = 76–100% sclerosis. The glomerulosclerosis score for each animal was then evaluated in the following manner: if among the total 100 glomeruli, 10 were of grade +1, 20 were of grade +2, 15 were of grade +3, and 5 were of grade +4, the final score was calculated as \((1 \times 10/100) + (2 \times 20/100) + (3 \times 15/100) + (4 \times 5/100)) \times 100 = 115\). Second, the degree of interstitial fibrosis was also evaluated using a similar semiquantitative scoring system. We examined approximately 20 non-overlapping cortical fields (magnification, \(\times 200\)) per animal and graded the severity of interstitial fibrosis from 0 to +4: 0 = normal, +1 = mild, +2 = moderate, +3 = severe, and +4 = very severe. The fibrosis score was then determined for each animal. For example, if out of the 20 fields examined, 5 were of grade +1, 3 were of grade +2, 2 were of +3, and 1 was of grade +4, the final score of fibrosis was calculated as \((1 \times 5/20) + (2 \times 3/20) + (3 \times 2/20) + (4 \times 1/20)) \times 100 = 105\). Third, COX-2-positive macula densa (MD) were counted in approximately 20 non-overlapping cortical fields (magnification, \(\times 200\)) per animal and expressed as the number per field. Fourth, renin-positive arterioles were evaluated using a method similar to that used to determine the number of COX-2-positive MD cells. The relationship between COX-2/renin expression and glomerular damage was then evaluated using PAS-counterstained sections as follows. The glomeruli with COX-2-positive MDs, COX-2-negative MDs, and renin-positive arterioles (level 1: narrow positive area, level 2: wide positive area) were selected at random, and the severity of sclerotic lesions of each glomerulus was graded from 0 to +4 described above.

Statistics

All quantitative data were expressed as the mean ± SE and were analyzed by Wilcoxon’s (Kruskal-Wallis) test and multiple comparisons [Tukey-Kramer honest significant difference (HSD) test] using the JMP 5.1 software program for Windows (SAS Institute Inc., Cary, NC, USA).

Results

Histopathology

Glomerular damage was observed in KK-\(A^\prime\) mice, and it gradually became more severe with age (Fig. 1A). Glomerular damage was characterized by the expansion of mesangial matrices. Mild nodular lesions were also observed in the damaged glomeruli. No apparent glomerular damage was observed in C57BL/6 mice (Fig. 1B). A gradual increase in glomerular sclerotic lesion scores was observed in KK-\(A^\prime\) mice (Fig. 2A), and these scores were significantly higher than those of C57BL/6 mice at 12, 16, and 20 weeks of age.

Tubulointerstitial damage, characterized by mononuclear cell infiltration and interstitial fibrosis, was observed in KK-\(A^\prime\) mice. The damage in KK-\(A^\prime\) mice was not severe until 20 weeks of age, and quantitation of interstitial fibrosis revealed no significant increases with age (Fig. 2B). Urinary casts were frequently observed within the tubular lumens of KK-\(A^\prime\) mice, and the associated tubules were commonly dilated.

Immunohistochemistry

COX-2-positive signals were observed in MD cells of the thick ascending limbs (TALs) in KK-\(A^\prime\) mice (Fig. 3A). The highest number of cells was detected at 8 weeks of age. This number decreased at 12 weeks of age, and then no changes were seen until 20 weeks of age (Fig. 2C). No positive COX-2 signals were detected in any kidneys of the C57BL/6 mice (Fig. 3B). In glomeruli with COX-2-positive MDs, grade of sclerotic lesions increased at 12 weeks of age, and then decreased slightly until 20 weeks of age (Fig. 2D). These grades were significantly higher than those with COX-2-negative MDs at 12 and 16 weeks of age.

Renin-positive signals were commonly detected in juxtaglomerular arterioles in all animals, and no differ-
Fig. 1. Light micrographs of kidneys. A: Sample from a 20-week-old KK-A' mouse. Two glomeruli show expansion of the mesangial matrices, and one glomerulus shows severe sclerosis. B: Sample from a 20-week-old C57BL/6 mouse. No lesions are observed. PAS stain. Bars: 100 µm.

Fig. 2. Results from quantitative analysis. A: Scores for sclerotic changes in glomeruli; B: scores for interstitial sclerosis; C: the number of COX-2 positive MDs; D: severity of sclerotic lesions in glomeruli with COX-2-positive or -negative MDs; E: the number of renin-positive arterioles; and F: severity of sclerotic lesions in glomeruli with level-1 or -2 renin-positive arterioles. KK: KK-A' mice. B/6: C57BL/6 mice. Data represents the mean ± SE. *: Significant differences in each age group (P<0.05, Tukey-Kramer HSD test). P values: significantly different as determined by Wilcoxon’s test. NS: no significant differences as determined by Wilcoxon’s test. ND: not detected.
ences were observed between KK-A′ and C57BL/6 mice in the localization of the positive signals (Fig. 3C and 3D). Significant changes in the number of renin-positive arterioles were observed in KK-A′ mice (Fig. 2E). They showed an increase at 16 weeks of age followed by a decrease at 20 weeks of age. Then numbers of renin-positive arterioles in the C57BL/6 mice showed no significant changes with age. The severity of sclerotic lesions in glomeruli with renin-positive arterioles showed significant but irregular changes, irrespective of the level of immunoreactivity (Fig. 2F). No significant differences between level 1 and level 2 renin-positive signals were detected in any age group.

**Discussion**

The histopathological findings of the present study clearly demonstrated the suitability of the KK-A′ mouse as a model of human type 2 DN. A previous study microscopically examined glomerular damage in diabetic KK-A′ mice at 20 weeks of age and demonstrated that the histopathological and immunohistochemical features of DN in this model resembled those in humans [11]. In the present study, expansion of mesangial matrices and nodular lesions, which are features of human diabetic glomerulopathy, were observed in diabetic KK-A′ mice, and gradual progression of glomerular sclerosis from 8 to 20 weeks of age was quantitatively demonstrated. Recently, progression of tubulointerstitial damage become to be considered the final pathway in the various types of nephropathy, including DN [19, 20]. In the present study, although tubulointerstitial damage was observed in the kidneys of diabetic KK-A′ mice, it was not very severe, and the interstitial fibrosis score did not increase significantly. Therefore, we consider that the KK-A′ mice in the present study did not suffer end-stage renal damage but showed early signs of DN. Albuminuria is a well known early sign of human DN. This clinical sign has already been confirmed in KK-A′ mice [11, 23], and the age-dependent gradual increase of the
COX-2 inhibitor were also demonstrated in studies using a COX-2 inhibitor [2]. The renoprotective effects of dothelial growth factor, and fibronectin were reduced by beta, plasminogen activator inhibotor-1, vascular en

treatment. Because of these important physiological roles, the functional roles of renal COX-2 have been well

investigated in rats [7, 8]. COX-2, which is constitutively expressed in the MD/TALs, plays important roles in the maintenance of kidney function, such as the regulation of RAS, medullary blood flow, and renal salt handling. Because of these important physiological roles, an increase in renal COX-2 expression was previously considered a renoprotective event in renal disease [6]. However, many recent studies have disproved this hypothesis. The renoprotective effect of a COX-2 inhibitor was initially reported in a study using a rat diabetes and hypertension model; in that study, expressions of renal injury mediators such as transforming growth factor-beta, plasminogen activator inhibitor-1, vascular endothelial growth factor, and fibronectin were reduced by a COX-2 inhibitor [2]. The renoprotective effects of COX-2 inhibitor were also demonstrated in studies using STZ-induced uninephrectomized rats, 5/6 nephrectomized rats, and hypertensive rats [5, 6, 14]. These renoprotective effects of the COX-2 inhibitor were not seen exclusively in rats but were also observed in a type 1 diabetes mouse model [21]; furthermore, the antiproteinuric effect of the COX-2 inhibitor in human patients was recently demonstrated [29]. These findings suggest that renal COX-2 expression is one of the pathological events of nephropathy. In the present study, although the number of COX-2-positive MD cells did not increase during DN progression, we suspected that renal COX-2 in KK-A' mice plays an important role in renal injury progression. So, we investigated the severity of sclerotic lesions in glomeruli with COX-2-positive MD cells, and demonstrated close a relationship between the severity of glomerular damage and expression of COX-2 in adjacent MDs. The most severe damage in the glomeruli with COX-2-positive MDs was detected at 12 weeks of age. Since a prior elevation in the total number of COX-2-positive MDs was observed at 8 weeks of age, we suggest that expression of COX-2 in MD cells is not a compensatory event induced by glomerular damage but an initial key event for the development of glomerular damage.

COX-2, which is expressed in renal MD cells, is known to regulate RAS via stimulation of renin produc

tion [8]. Activation of RAS plays a central role in the pathogenesis of DN in type 2 diabetes, and RAS inhibition drugs such as ACEi and ARB have been used to prevent DN [1]. On the basis of this evidence, we hypothesized that renal COX-2 expression in MD cells induces activation of RAS via an increase in renin production, and that this activation is one of the pathological mechanisms underlying DN development. However, the findings of renin immunohistochemistry analysis in the present study disprove this hypothesis. No differences were observed between diabetic KK-A' and control C57BL/6 mice in terms of localization of renin-positive signals. Although the numbers of renin-positive arterioles changed significantly in the KK-A' mice (increasing at 16 weeks and then decreasing at 20 weeks of age), no correlations between this change and COX-2 expression were detected. Although the increase in the number of renin-positive arterioles at 16 weeks of age might be related to the development of DN in KK-A' mice, this
elevation of renin was not considered to have been induced by COX-2 expression. The lack of association between renal COX-2 expression and DN renin production has also been demonstrated in rats [15, 26]. Administration of COX-2 inhibitor did not affect plasma or renal renin concentrations or renal renin mRNA expression in STZ-induced diabetic rats [15]. In other studies, it was demonstrated that COX-2 inhibitor did not affect blood pressure or urinary aldosterone excretion in hypertensive rats [26]. Therefore, we suspect that the mechanism by which COX-2 aggravates DN different from that of renin.

In the present study, the number of renin-positive arterioles in the KK-Δmice increased at 16 weeks of age. Although changes of renin expression in the juxtaglomerular arterioles were considered to affect the blood pressure by systemic RAS, the absence of changes in blood pressure (systolic, diastolic, and mean) has been demonstrated in the KK-Δmice from 12 to 20 weeks of age [11]. Therefore it was speculated that the elevation of renin expression at 16 weeks of age is considered related to up-regulation of intra-renal (local) RAS induced by hyperglycemia [4], and that elevation of local RAS is too little to induce increase in blood pressure in this model. Although we investigated the severity of sclerotic lesion in glomeruli with renin-positive adjacent arterioles, no relationships were demonstrated between the severity of glomerulosclerosis and the level of renin expression. Future studies focusing on RAS are necessary to clarify the detailed pathological mechanisms of DN in this model.

In summary, the present study investigated the kidneys of KK-Δmice from 8 to 20 weeks of age as a model of type 2 diabetes. Gradual progression of glomerulopathy was observed histopathologically, and this finding was considered an early sign of DN. Immunohistochemistry revealed a constant expression of COX-2 in MD cells from 8 weeks of age; however, this expression did not correlate with the changes in the number of renin-positive arterioles. Although renal COX-2 and renin are the key mediators of diabetic glomerulopathy, elevated production of each enzyme might be associated with the development and progression of DN through different mechanisms.

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### References


