Morphometrical Analysis of the Kidney from Nonobese Diabetic (NOD) Mice in the Non-Diabetic Stage

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ABSTRACT. The kidneys of non-diabetic NOD and wild type ICR mice were examined morphometrically at 3 and 6 months of age. Kidney weights and diameter of renal corpuscles of non-diabetic NOD mice were less than those of ICR mice. No lesions were observed in glomeruli or uriniferous tubules. Renin-positive areas were more common in NOD mice than in ICR mice, but no differences were detected in the Western blot analyses.

KEY WORDS: kidney, morphometry, non-diabetic NOD mouse.

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The nonobese diabetic (NOD) mouse is an established model of spontaneous type I diabetes mellitus [3]. This strain is derived from the Jcl:ICR cataract mouse strain, and the incidence of diabetes is higher in females than males [3]. After onset, this strain shows severe diabetic complications, which resemble those in human type I diabetes mellitus [13, 14]. Diabetic nephropathy is one of the most important complications [1, 19]. Several pathological investigations of diabetic nephropathy during the chronic or acute phases have been performed previously in NOD mice [7, 13, 14]. However, it remains unclear whether pathological changes characteristic of diabetic nephropathy are present before the onset of the disease in NOD mice. Therefore, in the present study, we performed morphometrical analysis of the kidney in non-diabetic NOD mice.

The present study was performed in accordance with Guidelines for Animal Experimentation of Kagoshima University, Japan. Three- and six-month-old female NOD/Shi Jic and wild type Jcl:ICR mice were used for the experiment (n=4/each group). Animals were housed in an open system room with a one-way airflow system (temperature 22 ± 1°C; humidity 55 ± 10%; light period 07:00 to 19:00; ventilation 12 cycles/hr) at the Division of Laboratory Animal Science, Research Center for Life Science Resources, Kagoshima University. Mice were given an autoclaved commercial diet (CE-2; Japan CLEA, Inc., Tokyo, Japan) and tap water *ad libitum*. Each mouse was monitored for glycosuria every-day using diagnostic papers (HI-TESPER-G; Eiken Chemical, Inc., Tokyo, Japan). None of the mice used in the present study exhibited glycosuria (>50 mg/dl) during the experimental period. Mice were sacrificed by exsanguinations of carotid arteries under anesthesia, using a mixture of ketamine and medetomidine. Kidneys and pancreas were quickly removed.

Right and left kidneys were weighed before fixation. Central slices from right and left kidneys, including the hilum, were cut perpendicular to the long axis. Slices from the left kidneys were fixed in 10% neutral buffered formalin at room temperature, and slices from the right kidneys and pancreata were fixed in Zamboni's solution. After routine embedding in paraffin, sections that included the elongate renal papillae were selected every 30 µm or serially. After deparaffinization, sections of the left kidney were stained with hematoxylin-eosin (HE) or periodic acid Schiff (PAS). Sections of the right kidney were used for PAS staining or immunohistochemical analysis. Immunostaining was performed using the VECTASTAIN *Elite* ABC Kit (Vector Laboratories, Inc., Burlingame, CA, U.S.A.). For detection of renin in kidneys, rabbit anti-human recombinant renin antiserum, supplied by Dr. Kazuo Murakami (University of Tsukuba, Japan), was used as the primary antibody (diluted 1:10,000), and biotinylated goat anti-rabbit IgG (H+L) (Vector Laboratories) was used as the secondary antibody (diluted 1:200). For detection of insulin in pancreata, guinea pig anti-porcine insulin antiserum (ready to use; DAKO Cytomation, Inc., Kyoto, Japan), which is cross-reactive with mouse insulin [6], was used as the primary antibody, and biotinylated goat anti-guinea pig IgG (H+L) (Vector Laboratories) was used as the secondary antibody (diluted 1:200). Immunoreactivity was detected with 0.025% 3,3'-diaminobenzidine-0.003% H₂O₂ solution, and counter staining was performed with hematoxylin.

Randomized morphometric analysis was performed according to previous methods [11, 12, 16–18]. Briefly, 1) the diameter of the cortical renal corpuscles [17], 2) index of mesangial expansion [12, 16], 3) number of PAS-positive granules in the proximal straight tubules (PSTs) [18] and 4) number of renin-positive areas [11] were recorded.

Values were expressed as the mean ± standard error (S.E.) for each group, and statistical significance was defined as p<0.05 using Student's *t*-test.

The remaining kidneys that had been stored at –80°C

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Fig. 1. Detection of insulin by immunohistochemistry in the pancreas. (a): 6-month-old NOD mouse. (b): 6-month-old ICR mouse. Bar=40 μ m.

were homogenized in 25 mM Tris-HCl-buffered saline (pH7.4) containing 1 mM phenylmethanesulfonyl fluoride (Sigma Chemical, Co., Missouri, U.S.A.), 1 mM EDTA-2Na and 3 mg/ml of leupeptin (Sigma), and then centrifuged at 14,000 rpm for 10 min. Routinely prepared samples (25 μ g of proteins) were separated by SDS-PAGE, then transferred to nitrocellulose membranes. After blocking, membranes were incubated with anti-recombinant renin antiserum, diluted 1:10,000, overnight at 4°C. Peroxidase

conjugated anti-rabbit IgG (Amersham Biosciences, Inc., New Jersey, U.S.A.), diluted 1:5,000, was used as the secondary antibody, and immunoreactive bands were detected by an ECL Kit (Amersham Biosciences).

Although immune-mediated insulinitis in the NOD mouse strain begins at 4 weeks of age, it is well known that the onset of diabetes is not observed until terminal B cells loss [8]. The NOD mice used in the present study also exhibited mild insulinitis, but no clinical signs of diabetes were observed and many insulin-positive B cells remained (Fig. 1).

Wet kidney weights of NOD mice were significantly lower than those of ICR mice at 3 and 6 months of age (Fig. 2A-a; $P<0.05$). The ratio of kidney weight to body weight of NOD mice was significantly lower than that of ICR mice at 3 months (Fig. 2A-b; $P<0.05$). The diameter of cortical renal corpuscles in the NOD mice was significantly smaller than that in ICR at 3 and 6 months (Fig. 2B-a; $P<0.05$). The index of mesangial expansion was low in all mice, and no significant differences were detected between NOD and ICR mice (Fig. 2B-b; $P<0.05$). Renal hypertrophy and glomerulosclerosis are well known events of diabetic nephropathy in diabetes mellitus [2, 10, 13, 14, 19]. The former

Fig. 2. [A]: Changes in the kidney weights. (a): Total kidney weights. (b): The ratio of total kidney weights to body weight. [B]: Changes in renal corpuscle parameters. (a): Diameter of renal corpuscles. (b): Index of mesangial expansion. [C]: The number of PAS-positive granules in the proximal straight tubules (PSTs). [D]: The number of renin-positive areas. Values are mean $\pm$ S.E.M. for each age group. *: significant difference vs ICR in the same age group ($P<0.05$).

is a pathological feature of the early stage and the latter is a feature of the chronic stage. These events have been demonstrated also in the NOD mice [13, 14]. Our present results demonstrated that renal hypertrophy and glomerulosclerosis were not present in the non-diabetic stage. This evidence suggested that renal pathological changes in the diabetic-NOD mouse are not age-dependent changes but diabetic changes.

No structural differences between NOD and ICR mice were observed in any segments of the uriniferous tubules. In general, PAS-positive granules are a female-specific feature of the PSTs, and they have been identified as large-sized lysosomes [15, 18]. In addition, our recent report showed that abundance of these PAS-positive granules increased during the acute diabetic stage in NOD mice (mean age of onset; 154 days) [7]. However, whether these changes during the acute stage of NOD were age- or diabetes-dependent had remained unclear. In the present study, PAS-positive granules were observed in the PSTs of NOD mouse as well as those of ICR mice (Fig. 3). Figure 2C shows the number of PAS-positive granules in the PSTs. Quantification of our results demonstrated that the number of PAS-positive granules in the PSTs remained constant in the NOD but increased in the ICR at least in 3- to 6-month-old mice. Therefore, the increase in PAS-positive granules during the acute stage in NOD mice is not an age-dependent but a diabetes-dependent pathological change.

Renin-positive reactions were commonly detected in juxtaglomerular cells of afferent arterioles and rarely in glomeruli. The localization of renin-positive reactions did not differ between NOD and ICR mice. However, the number of renin-positive areas in NOD mice was significantly higher than that in ICR mice at 3 and 6 months (Fig. 2D). Activation of the renin-angiotensin system (RAS) is recognized as an important risk factor in diabetic nephropathy [5, 9], and the present findings suggest that RAS activity during the non-diabetic stage in NOD mice is higher than that in the wild type ICR. However, Western blot analysis could not support this hypothesis. Briefly, renin immunoreactive bands with a molecular weight of approximately 39 kDa were detected with equal densities in homogenates from NOD and ICR kidneys (Fig. 4.). Therefore, further investigations of RAS activity in non-diabetic NOD mice and its importance as a risk factor for the development of diabetic nephropathy are necessary.

In conclusion, we morphometrically analyzed the kidneys of non-diabetic NOD mice. The present results revealed that the kidneys of non-diabetic NOD mice were smaller than those of wild type ICR. No lesions were observed in the glomeruli and uriniferous tubules. Although the number of renin-positive areas was higher in NOD mice than in ICR mice, high RAS activity was not demonstrated by Western blot analysis. The findings of the present study provide significant morphological data for investigations of the pathogenesis of diabetic nephropathy using the NOD model.

Fig. 3. Light micrograph of the outer medulla. (a): 6-month-old NOD mouse. (b): 6-month-old ICR mouse. Many cytoplasmic granules are observed in the proximal straight tubular epithelium of both strains. PAS stain. Bar=20 μ m.

Fig. 4. Western blot analysis of renin in the kidneys. Lane 1: 3-month-old NOD mice, Lane 2: 3-month-old ICR mice, Lane 3: 6-month-old NOD mice, and Lane 4: 6-month-old ICR mice. No apparent differences are seen between NOD and ICR mice.

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