Global heterogeneity of glomerular volume distribution in early diabetic nephropathy

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Background. Morphologic characteristics in early stage of nephropathy of noninsulin-dependent diabetes mellitus (NIDDM) have not been determined despite the fact that diagnosis in this stage of the disease is important for the prognosis. We hypothesized that heterogeneity in glomerular volume-distribution may be a sensitive index of early stage of diabetic nephropathy in NIDDM.

Methods. In spontaneous diabetic rats [Otsuka Long-Evans Tokushima Fatty (OLETF) rat (N = 5)] of 27 to 28 weeks, an experimental model of early diabetic nephropathy in human NIDDM and age-matched control rats [Long Evans Tokushima Lean (LETO) rat (N = 5)], we completely filled the kidney with contrast medium. Glomeruli were visualized as three-dimensional images using x-ray micro-computed tomography (micro-CT). Glomerular volumes (N = 400 in each kidney) were directly measured and evaluated as absolute volume and normalized values to kidney weight and body weight. Scattering of glomerular volume-distribution was evaluated as coefficient variation (CV) (SD/mean).

Results. The CV was significantly larger in OLETF rat (0.195) comparing to LETO rat (0.146, P < 0.01). This difference was even consistent under the normalization to kidney weight and body weight. Absolute glomerular volume was larger in OLETF rat compared to LETO rat (P < 0.005); however, when glomerular volume was normalized, this variable was comparable between two groups.

Conclusion. We visualized three-dimensional glomerular images in the early stages of diabetic nephropathy using micro-CT and quantified the heterogeneity in glomerular volume distribution throughout the cortex by direct measurement of the individual. We propose that heterogeneity in glomerular volume distribution is a sensitive parameter to ascertain early diabetic nephropathy in NIDDM.

Key words: glomerular volume, heterogeneity, diabetes mellitus, nephropathy, morphology, micro-CT, three-dimensional visualization.

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and a synchrotron radiation micro-CT. The former was mainly used to compile image data file, whereas the synchrotron radiation micro-CT at Japan Synchrotron Radiation Research Institute that provides the world most intense collimated beam of monochromatic x-ray was used to validate the image quality and volumetric analysis.

METHODS

Animal supply

Spontaneous diabetes mellitus rats [Otsuka Long-Evans Tokushima Fatty (OLETF) rat] and age-matched control rats [Long-Evans Tokushima Lean (LETO) rat] were provided by Otsuka Pharmacology Co., Ltd (Tokushima, Japan).

Contrast medium

We used modified contrast medium originally prepared in our recent studies [15]. Briefly, this contrast medium consists of (1) 40% vol/vol (in distilled water) of BaSO₄ (Baritgen zol, φ 0.7 μm BaSO₄ particles) (Fushimi Co., Ltd., Marugame, Japan), which allows vivid contrast of x-ray absorption in micro-CT imaging, (2) 20% vol/vol of India ink (Saga Kokyu Nosen, Kaimei Co., Ltd., Osaka, Japan) which enables verification of filling using stereomicroscope, and (3) 8% wt/vol of gelatin (Nacional Tesque Inc., Kyoto, Japan) which enables complete filling of the vasculature. The contrast medium is warmed at 42°C for the renal administration and is intensively solidified by cold saline exposure (0 to 2°C).

Sample preparation

Our experimental procedures and protocols were conducted according to the institutional guidelines approved by the Animal Research Committee of Kawasaki Medical School (No. 99318). All rats were ordinarily fed until the day of the experiment. Twenty-seven to 28-week-old OLETF rats (male 639 ± 31 g body weight, N = 5) and age-matched LETO rats (male 497 ± 21 g body weight, N = 5) were used for the experiments. The diabetic phenotype of OLETF rat has previously been extensively evaluated: (1) nearly 100% incidence of diabetic syndrome (hyperglycemia, hyperlipidemia, etc.) after 25 weeks [14], and (2) proteinuria and albuminuria starting to be observed after 30 weeks [16]. Thus, we assumed that OLETF rats at 27 to 28 weeks old were in the early stages of diabetic nephropathy. To determine that differences in glomerular volume and coefficient variation (CV) were due to the manifestations of diabetes, we examined the morphometric parameters in 7-week-old OLETF and LETO rats (Table 1). OLETF rats at the age of 7 weeks are not yet diabetic.

Rats were anesthetized using intraperitoneal administration of pentobarbital (60 mg/kg) following by intravenous heparin administration (500 U). Respiration was controlled by ventilator (Model 7025) (Biological Research Apparatus, Comerio, Italy) with 100% oxygen inhalation via tracheostomy. After laparotomy, the superior mesenteric artery, inferior mesenteric artery, and celiac trunk were ligated by 5-0 silk. The lower abdominal aorta was retrograde cannulated (21 G catheter) for the sampling of blood glucose measurement and for fluid administration. The abdominal aorta just proximal to the left renal arterial branching was ligated, then saline was selectively perfused into a left kidney for 3 to 5 minutes to completely wash out red blood cells. The perfusate was then changed to 10% glucose solution for 3 to 5 minutes. After punctuation of the left renal vein, contrast medium (42°C) was administered into the left kidney through the aortic cannula at a pressure of 250 mm Hg. After 20 to 30 seconds, cold saline (0 to 2°C, total of 500 mL) was gently poured into the abdominal cavity to solidify the contrast medium. Finally, the left kidney was removed and fixed in the 4% paraformaldehyde overnight. Since the left kidney was filled with contrast medium and fixed, the right kidney was removed and weighed to normalize glomerular volume to the kidney weight.

Three-dimensional imaging using micro-CT

The fixed kidney was cut along the coronal axis and a small column (4 mm in diameter) from cortex to medulla was punched out in the mid-portion of the kidney. The sample was transferred to a polyethylene tube filled with phosphate buffer solution for micro-CT observation. One sample column was obtained from each kidney (N = 5 in OLETF rats and LETO rats, respectively) for micro-CT observation. In one kidney from each strain, another tissue column was obtained for synchrotron radiation micro-CT observation.

Renal tissues were observed using micro-CT (Elescan, NX-NCP-C80-I) (Nittetsu ELEX, Kitakyushu, Japan). Specifications of the micro-CT were described in our previous study [15]. Briefly, the specifications are (1) x-ray image intensifier has 72 × 54 mm²; (2) charged-coupling device (CCD) camera has 640 × 480 pixels; (3) geometrically, the pixel size under the maximum magnification (×20.0) is 5.6 μm; and (4) reference width lines of 14.7 μm in the US Air Force Target Scale were discriminated as the maximal resolution. In the present study, the pixel size of 9.6 μm (×11.8) was adopted to focus on the signal of the vessels larger than capillaries, while defocusing capillaries. CT-image for each slice (9.6 μm in thickness) was obtained by 600 projections with eight times accumulation at 30 keV, 100 μA.

To validate the image quality and vascular volume analysis by the micro-CT, additional renal samples were
Table 1. Glomerular volumes and its coefficient variation (CV) in Otsuka Long-Evans Tokushima Fatty (OLETF) and Long-Evans Tokushima Lean (LETO) rats

<table>
<thead>
<tr>
<th>Sample</th>
<th>OLETF</th>
<th>LETO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glomerular volume $\mu m^3$</td>
<td>2,115,482, 2,150,032, 2,552,292, 2,206,656, 2,339,734, 2,272,839</td>
<td>1,325,300, 1,431,004, 1,698,134, 1,815,527, 1,721,030, 1,598,199</td>
</tr>
<tr>
<td>SD</td>
<td>407,222, 409,284, 543,620, 384,863, 483,354, 445,669</td>
<td>202,920, 208,239, 218,752, 276,370, 258,034</td>
</tr>
<tr>
<td>CV</td>
<td>0.192, 0.190, 0.213, 0.174, 0.207, 0.195</td>
<td>0.153, 0.146, 0.129, 0.152, 0.150, 0.146</td>
</tr>
<tr>
<td>SD</td>
<td>407,222, 409,284, 543,620, 384,863, 483,354, 445,669</td>
<td>39,402, 45,269, 43,317, 55,274, 51,096</td>
</tr>
<tr>
<td>CV</td>
<td>0.192, 0.190, 0.213, 0.174, 0.207, 0.195</td>
<td>0.153, 0.146, 0.129, 0.152, 0.150, 0.146</td>
</tr>
<tr>
<td>Glomerular volume kidney weight $\mu m^3/100g$</td>
<td>907,932, 892,129, 1,257,287, 676,888, 921,155, 931,078</td>
<td>716,379, 1,022,145, 1,239,514, 1,315,599, 1,089,260, 1,076,579</td>
</tr>
<tr>
<td>SD</td>
<td>174,773, 169,827, 267,793, 118,056, 190,297, 184,149</td>
<td>109,687, 148,742, 159,673, 200,268, 163,312</td>
</tr>
<tr>
<td>CV</td>
<td>0.192, 0.190, 0.213, 0.174, 0.207, 0.195</td>
<td>0.153, 0.146, 0.129, 0.152, 0.150, 0.146</td>
</tr>
</tbody>
</table>

Glomerular capillary volumes in 27 to 28 weeks OLETF rats and age-matched LETO rats were directly measured using micro-computed tomography (micro-CT). Total of 400 glomeruli were measured in each specimen. *P < 0.005 vs. LETO; **P < 0.01 vs LETO.

Visualized using the synchrotron radiation micro-CT [15, 17]. SPring-8 (http://www.spring8.or.jp) that provides an intense collimated beam of nearly parallel monochromatic x-ray. X-ray image was detected on the fluorescent screen lens coupling CCD camera detector with 6 $\mu m$ in the pixel size. Reference width lines of 8.8 $\mu m$ in the US Air Force Target Scale were discriminated as the maximal resolution. CT-image was obtained by 360 circular projections in 1024 x 1024 pixels. X-ray energy was 17 keV.

Validation study of contrast medium filling and image quality of micro-CT

After obtaining the tissue specimen (column) the remaining kidney was embedded in 12% gelatin block and placed in 4% paraformaldehyde overnight. This sample block was sliced into 100 $\mu m$ thickness using a vibrating microtome (VT1000S) (Leica Inc., Heidelberg, Germany), then inspected using stereomicroscope (Wild MZ8) (Leica Inc.) to ensure that the contrast medium completely filled the renal microvasculature.

To validate the image quality of the micro-CT, a supplemental observation was performed using a sample column (in LETO rat) after completing the micro-CT visualization. This sample was picked out from the polyethylene tube, embedded in 12% gelatin block, fixed again, sliced with 100 $\mu m$ thickness, and observed using stereomicroscope. Digital images (DXM1200) (Nikon, Tokyo, Japan) of an optional cross-section were compared with the micro-CT images in the similar layer.

Glomerular volumetric analysis

The micro-CT images were processed to optimize for vascular enhancement by referring to the signal histogram and digital images were converted into the stack files (NIHimage1.62) for volume rendering (Voxblast2.2) and three-dimensional animation (Quick-Time J-4.1 available at the following URLs: http://www.blackwellpublishing.com/products/journals/suppmat/ki66/ki66-2/ki66-2appendix1.qt; and http://www.blackwellpublishing.com/products/journals/suppmat/ki66/ki66-2/ki66-2appendix2.qt). Total of 400 glomeruli throughout the cortex were randomly selected and individual glomerular volume was measured as follows. The image data files were processed (MATLAB5.2) to clearly distinguish the glomerular signal from the noise derived from surround peritubular capillaries and digital noises. The signals of visible afferent and efferent arterioles connected to the glomerulus were manually erased (NI-Himage1.62). An individual glomerulus was displayed
on the computer monitor as a rotated three-dimensional animation to verify whether the targeted glomerulus was disconnected from its afferent and efferent arterioles. Glomerular volume was analyzed by calculating glomerular area in each CT slice, followed by scanning this process along depth direction. Glomerular volume was evaluated as absolute value ($\times 10^6 \mu m^3$) and normalized to the kidney weight ($\times 10^6 \mu m^3/g$) and the body weight ($\times 10^6 \mu m^3/100 g$). Scattering of the glomerular volume distribution was expressed by $CV = SD/mean$.

**Statistical analysis**

All data are expressed as mean ± SD. Glomerular volumes were compared between OLETF rats and LETO rats using one-way analysis of variance (ANOVA) followed by Fisher pairwise least-significant difference test using StatView 5.0 PPC. Comparison of mean and CV of glomerular volumes both in absolute and normalized values, blood glucose, kidney weight, and body weight were performed by unpaired $t$ test. Statistical criterion was defined as $P < 0.05$.

**RESULTS**

**Validation of contrast medium filling and micro-CT image quality**

Contrast medium consistently filled the entire microvasculature of the kidney, including all glomeruli, arterioles, and peritubular capillaries. The contrast medium (with India ink’s vivid black color) was obvious and no filling defects were observed by the stereomicroscopic observation. Figure 1 shows representative stereomicroscopic images (Fig. 1A, low magnification and Fig. 1B high magnification, 100 $\mu m$ thickness-sample, black-white reverse images) and corresponding level of micro-CT images (Fig. 1C, low magnification and Fig. 1D, high magnification, 10 $\mu m$ thickness, CT reconstruction image) in a LETO rat. There was striking similarity of the discriminated capillaries between the micro-CT image (Fig. 1C) and the stereoscopic image (Fig. 1A), a little mismatch was observed, which was probably due to unavoidable displacements by the mechanical slice of the microtome. Importantly, corresponding glomerular images were consistent (Fig. 1B and D). Our micro-CT imaging was assumed to have enough quality for glomerular evaluation.

**Micro-CT image and glomerular volumetric study**

Rotational representative three dimensional micro-CT images of the OLETF rat and LETO rat are shown (Quick Time movies I and II). Entire glomeruli were clearly visualized. Five representative three-dimensional images of the glomeruli with higher magnification out of both samples are shown in Figure 2 (Fig. 2A, OLETF rat and Fig. 2B, LETO rat). In OLETF rat, glomerular stereoscopic structure was characterized by higher irregularity. In LETO rat, on the other hand, glomeruli were characterized by similar spheroid or elliptic configurations.

The volumetric analysis of glomeruli ($N = 400$ for each kidney) is shown in Figure 3. Glomerular volume was shifted rightward (larger volume) and the distribution was expanded in the OLETF rat (Fig. 3A) compared to the LETO rat (Fig. 3B). In all samples ($N = 5$), the glomerular volume was significantly larger in OLETF than LETO rat ($P < 0.0001$, ANOVA). Mean volume in the OLETF rat ($2.27 \pm 0.18 \times 10^6 \mu m^3$) was
When the glomerular volumes were normalized to the kidney weight, however, differences between both strains were not observed \((0.93 \pm 0.21 \times 10^6 \, \mu m^3/g)\) in OLETF rats and \(1.07 \pm 0.23 \times 10^6 \, \mu m^3/g\) in LETO rats, \(P = 0.35\). Also, the normalization to the body weight showed comparable results between both strains \((0.36 \pm 0.03 \times 10^6 \, \mu m^3/100 \, g\) weight in the OLETF rats and \(0.32 \pm 0.04 \times 10^6 \, \mu m^3/100 \, g\) weight in the LETO rats, \(P = 0.16\). Nevertheless, heterogeneity of glomerular volume in the OLETF rat still showed a significantly larger value than the LETO rat even when normalized to the kidney weight and the body weight \((0.195\) in OLETF rat and 0.146 in LETO rat, \(P < 0.01\), respectively).

The glomerular volumes of OLETF and LETO rats at 7 weeks of age were \(0.54 \pm 0.08 \times 10^6 \, \mu m^3\) and \(0.69 \pm 0.04 \times 10^6 \, \mu m^3\), respectively. These sizes were only \(\sim\) one third smaller than those of 27 to 28 weeks. The CV values of both OLETF \((0.295)\) and LETO \((0.255)\) rats in 7 weeks were larger than those of 27 to 28 weeks, however, no statistical difference in CV was observed between two strains in this young age.

The general database of both strains of rats was as follows: (1) blood glucose value during surgery was significantly higher in OLETF rats \((617 \pm 161 \, mg/dL\) during surgery\) than LETO rats \((379 \pm 66 \, mg/dL\) during surgery, \(P < 0.05\), (2) the kidney weight was significantly larger in OLETF rats \((2.5 \pm 0.5 \, g)\) than LETO rats \((1.5 \pm 0.2 \, g, P < 0.05)\), and (3) the body weight was significantly larger in OLETF rats \((639 \pm 31 \, g)\) than LETO rats \((497 \pm 21 \, g, P < 0.0001)\), which were all consistent with those in similar age of OLETF rat and LETO rat in the previous report \([18]\). OLETF rats at 7 weeks had a body weight of 225 \pm 18 \, g and a blood glucose concentration of 158 \pm 37 \, mg/dL. Age-matched LETO rats had comparable body weight \((211 \pm 10 \, g)\) and blood glucose \((160 \pm 28 \, mg/dL)\) to the OLETF rats \((NS)\).

**DISCUSSION**

In the present study, we assessed indices of glomerular structure using three-dimensional visualization to characterize an early stage of diabetic nephropathy. We found that the OLETF rat showed significantly higher heterogeneity in glomerular volume distribution comparing to the age-matched LETO rat. This difference was apparent even after normalization to kidney weight and body weight. Interestingly, normalized glomerular volumes on the average were comparable between OLETF rat and LETO rat. We, therefore, propose that heterogeneity in glomerular volume distribution is a sensitive morphologic parameter to ascertain early state of diabetic nephropathy in NIDDM.

There has been a clinical contradiction in nephropathy in NIDDM versus that in insulin-dependent diabetes
mellitus (IDDM). In IDDM, nondiabetic nephropathy is rare and the glomerular enlargement can predict the development of overt nephropathy [8, 9, 19, 20], but does not occur in NIDDM. Many investigators have tried to determine some characteristics of morphologic abnormality in NIDDM. However, NIDDM patients showed much variability in glomerular enlargement [11] with highly variable scoring in histologic abnormality (glomerular sclerosis, glomerular basement membrane thickening, and Bowman’s capsule thickening [11, 12]). This variability resulted in failure to reach a simplified clear answer regarding renal changes in NIDDM. It was, however, remained to investigate quantitative evaluation of global heterogeneity in glomerular pathomorphology by accurate/direct measurement (thus, not by estimation) of glomerular volumes all over the kidney. In the present study, we demonstrated that a simple and appropriate parameter, CV of glomerular volume distribution, enabled detection of the morphologic alterations in NIDDM nephropathy, even at an early stage. It was crucial that sufficient numbers of glomeruli (N = 400) from one renal sample were evaluated for the measurement of CV in order to have adequate power to detect possible statistical significance between diabetic and nondiabetic kidneys. Our preliminary study showed smaller numbers of glomeruli were not sufficient for the comparison [i.e., P value was 0.053 (NS) if N = 50, whereas P value slightly improved to 0.043 if N = 100]. Micro-CT enabled performance of the volumetric study and analysis of sufficient numbers of glomeruli, but this measurement would be much more difficult to accomplish using conventional histologic evaluations.

In the present study absolute glomerular volume was significantly larger in OLETF rats at 27 to 28 week of age (about 7 months) compared to the age-matched LETO rats. Uriu et al [18] demonstrated a significant increase in glomerular filtration rate in OLETF rats of 5 to 10 months old compared to the age-matched LETO rats. Thus, glomerular enlargement in OLETF rats in the present study corresponded to hyperfiltration-state. However, the difference in absolute volume between OLETF rats and LETO rats was eliminated after normalization to the kidney weights, resulting in failure to detect a change in glomerular volume in diabetic nephropathy. This finding was consistent with previous consequences based on histologic evaluations [18, 21, 22]. Specifically, an increase in estimated glomerular volume was accompanied with increase in total kidney mass under the hyperfiltration-state in diabetes mellitus [23, 24].

**Study limitation**

Our purpose was to evaluate global anatomical alterations of the renal microcirculation. Therefore, we performed our specific complete filling method using higher concentration of barium sulfate suspension and with extremely high perfusion pressure for the injection. We needed to use higher barium sulfate-concentration (40%, thus, higher viscosity) compared to the original concentration (20%) used in a coronary microvascular evaluation [15]. Forty percent was the minimum concentration to obtain adequate glomerular enhancement effect and better signal/noise ratio for the volumetric analysis. We employed 250 mm Hg as the perfusion pressure to inject the contrast medium into entire glomeruli. Since the renal microvascular volume was assumed to depend on the perfusion pressure, we believed that apparent heterogeneity in glomerular volume data was not determined by heterogeneity in perfusion distribution (due to insufficient pressure) but the anatomic alteration per se. Nevertheless, despite our specific preparation by higher viscosity of the contrast medium/higher perfusion pressure, averaged diabetic glomerular volume in the present study (2.27 × 10^-6 m³) was consistent with those by conventional histologic estimation in OLETF rats (2.0–2.5 × 10^-6 m³ [18]) and also in human NIDDM (ranged from 1.4 × 10^-6 m³ [19] to 2.4 × 10^-6 m³ [25]). We believe it is fair to compare the glomerular volumes between the samples under the identical preparation/conditions.

Although an increase in CV was demonstrated to correlate with diabetic nephropathy, there remain concerns for the diagnosis of the disease using this variable. The difference in body size between both groups may affect the CV value, but there are no background data from which to make any prediction. Because of much faster growing rate of body mass in OLETF [14], age difference (i.e., younger OLETF vs. older LETO) in the body weight matching may further complicate results because ages would be unequal. We additionally examined glomerular volume and its CV of LETO and OLETF rats at age of 7 weeks, prior to the onset of diabetes (body weights were comparable). CV values between two strains showed no statistically difference. However, both CV values were larger than that of 27 to 28 weeks of age in early phase of diabetes mellitus. Since absolute glomerular size of both strains were quite smaller than that of 27 to 28 weeks (only ∼1/3), the larger heterogeneity of CV in 7 weeks may be commonly caused by heterogeneity in individual “glomerular capillary growth” in young age. Nevertheless, heterogeneity of glomerular size appears to be pathophysiologically important in adult rats and predictive of diabetic nephropathy.

Finally, evaluation of global heterogeneity in glomerular volume distribution throughout the cortex using micro-CT allows new insight into early changes prognostic further renal pathomorphologic progression. Although spatial resolution and technology of most presently used imaging system are inadequate to make similar measurement to what we report, we emphasis that
imaging technologies are advancing. Thus, we believe this variable and method may have future clinical application by early diagnosis of occult nephropathy.

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