Repeated Streptozotocin Injections Cause Early Onset of Glomerulosclerosis in Mice

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Abstract: Diabetic nephropathy (DN), a major cause of end-stage chronic renal failure, is histologically characterized by glomerulosclerosis. To investigate the molecular mechanisms of DN, it is important to establish a stable model of glomerulosclerosis in mice, because genomic manipulation techniques (such as gene destruction or transgene insertion) are well established in rodent species. In this study, we found that repeated administrations of streptozotocin led to early onset of glomerular sclerotic lesions in C57BL/6 mice, accompanied with renal dysfunction. During the natural course of DN, glomerular endothelial cells decreased at 10 weeks after the start of streptozotocin-injections, whereas myofibroblastic mesangial cells became evident. Our results provide an animal tool to elucidate the molecular mechanisms of DN, for example to investigate vascular pathology in diabetic glomerular diseases.

Key words: diabetic nephropathy, glomerulosclerosis, mice

Diabetic nephropathy (DN) is now one of the most important causes of chronic renal failure [11]. Glomerulosclerosis is the histopathological hallmark of DN and may be an initial determinant for predisposition to renal dysfunction [22]. To develop a strategy for the treatment of DN, streptozotocin (STZ)-injected rats were used as an animal model of human DN. Actually, diabetic rats are useful especially for pharmacological studies of DN [18, 19, 23], because rats have certain merits in clinical and physiological evaluations of chronological observation. In addition, it is also important to elucidate a molecular mechanism(s) whereby glomerulosclerosis progresses under diabetic conditions. In this regard, genetic manipulations (such as transfection of a transgene(s) or disruption of genomic DNA) would shed light on elucidating the molecular basis of developing or developed DN. Because these genomic techniques are usually performed on mice (rather than rats), it is critical to establish a mouse model of DN. However, there is still controversy as to whether STZ induces glomerular sclerotic lesions in mice [1, 2]. In the current study, we found that repeated administrations of STZ in mice provoked earlier onset of glomerulosclerosis in mice (with a change in glomerular vascular cell components) than previously reported [1, 2].

We used the C57BL/6 mouse strain for the following reasons: 1) this strain is susceptible to STZ, leading to...
onset of atherosclerosis [9], an analog of glomerulosclerosis; and 2) the genomic background of this strain is popular for the generation of transgenic or gene-disrupted mice [1, 2]. Thus, we attempted to induce hyperglycemia in 8-week-old female C57BL/6CrSlc mice (Nihon Slc, Hamamatsu, Japan) under a SPF condition, based on the method of Chen et al. [2], with modifications: after food fasting for 24 h, we administrated STZ (Nacalai, Kyoto, Japan) at a dose of 120 mg/kg/day (i.p.) for the initial 2 days, and thereafter, at a dose of 80 mg/kg (i.p.) for the subsequent 2 days. Additional injections depended on changes in urine color: when the color remained yellowish at the 4th injection of STZ, we temporarily stopped the injection for 2 days, because of the time lag between STZ exposure and onset of hyperglycemia. After withdrawal, when the color of the urine turned water-like, no additional administration was needed. In contrast, when the color was still yellowish, two or three injections of STZ (80 mg/kg/injection) were required. As a result, approximately 70% of mice manifested severe hyperglycemia (plasma glucose level > 600 mg/dl) within one week after the last injection of STZ, whereas 20% of the animals died of severe dehydration, with loss of body weight. The remaining mice (with slight hyperglycemia) were removed from the present study, because they did not manifest diabetic glomerulopathy and renal dysfunction in the current experimental period (unpublished data).

To determine the natural course of DN, STZ-treated mice were subjected to autopsy at 2, 6, and 10 weeks after STZ-injections. As a control, age-matched intact mice were used to compare pathological or physiological conditions at the same time points. Each scheduled autopsy included 6 STZ-treated mice and 6 intact mice as diabetic and non-diabetic mice, respectively. At necropsy, plasma was collected, frozen and stored at –80°C until use. Plasma glucose levels were determined with a kit (Glucose B test Wako: Wako, Osaka, Japan), which is clinically used for human patients. Blood urea nitrogen (BUN) levels were determined by the urease-indophenol method, using a kit (Urea B test Wako: Wako) [15–17]. Renal tissues were fixed in 70% ethanol at 4°C for 24 h for renal histopathology [15–17]. The tissue segments were paraffin-embedded and cut at a thickness of 4 μm. The tissue sections were de-paraffined and stained in hematoxylin eosin (HE) solution. To detect glomerular changes, the mesangial expansion score was determined in at least 30 glomeruli in the HE-stained sections, according to an established method [20]. The remaining sections were subjected to the following immunohistochemical procedures. To detect extracellular matrix (ECM) protein (a marker for sclerosis/fibrosis [15–17]), anti-mouse fibronectin rabbit IgG (Chemicon, Temecula, CA, USA) was applied to the sections, followed by an ABC immunoperoxidase method with a kit (Vectastain Elite: Vector Lab., Burlingame, CA, USA) [15–17]. To evaluate glomerular endothelial cellularity, anti-human von-Willbrand factor (vonWF) (Dako Japan, Kyoto, Japan) and anti-mouse CD31 rat IgG (BD Biosiences, San Jose, CA) were used as primary antibodies, followed by the ABC technique as mentioned [7]. Furthermore, anti-α-smooth muscle actin (α-SMA) IgG (Dako Japan) was used as a cell marker to detect activated mesangial cells, which had undergone a phenotypic switch (i.e., transdifferentiation) to myofibroblasts [4, 15, 17]. To semiquantify degrees of glomerular lesions, tuft staining scores were calculated, based on the following grades: 0 = absent, 0.5 = trace, 1 = positive of <25% of total tuft, 2 = positive of 25–49% of total tuft, 3 = positive of 50–75% of total tuft, and 4 = positive of >75% of total tuft [15]. The score was expressed as a mean value of the grades, which were determined on at least 30 glomeruli per mouse.

Prior to evaluating glomerular changes, we initially characterized the clinical features in the diabetic mice. As described above, hyperglycemia was sustained at a considerably high level (plasma glucose levels > 600 mg/dl) after repeated injections of STZ (Table 1). During the present experimental period, no diabetic animal died, but some mice (<30% of total animals) manifested abdominal ascites or submandibular skin ulcer/abscess, while other mice showed alopecia on their back or head, with a loss in body weight. These dermatological changes may occur as a reflection of hyperglycemia-related microcirculation failures. Of note, BUN levels gradually increased up to 10 weeks after the STZ injection (Intact: 25.3 ± 3.22 mg/dl vs. 10W: 40.2 ± 6.22, p<0.01), suggesting onset of renal dysfunction during the progression of DN. Using the current STZ protocol, we have performed more than 10 experiments in vivo, in which diabetic nephropathy (including hyperglycemia, glomerulopathy and renal
We determined if glomerulosclerosis is found in the STZ-induced diabetic mice. Histopathological examinations revealed that mesangial expansion was frequently noted as early as 6 weeks after the STZ-injections (Fig. 1A). Furthermore, glomerular hypertrophy (as evidenced by an increase in tuft diameter) became obvious in the diabetic mice. Consistently, the mesangial expansion score significantly increased in diabetic mice from 6 weeks after the STZ-injections (Fig. 1A). In this regard, ECM protein (such as fibronectin) is known to increase not only along the glomerular basement membrane (GBM) but also in the mesangial areas in glomeruli under chronic glomerular dysfunction.

### Table 1. Clinical findings of the diabetic mice after completion of STZ injections

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Weeks after STZ injections</th>
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<tr>
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<tr>
<td>Plasma glucose (mg/dl)</td>
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<tr>
<td>diabetic</td>
<td>186.3 ± 22.1</td>
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<tr>
<td>non-diabetic</td>
<td>31.3 ± 5.7</td>
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<tr>
<td>BUN (mg/dl)</td>
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</tr>
<tr>
<td>non-diabetic</td>
<td>186.3 ± 22.1</td>
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a: glucose levels in plasma obtained from 24-h-fasting mice. b: mean ± S.D. (n=6). For abbreviations see text.

*Fig. 1.* Time course of glomerular sclerotic lesions in STZ-injected mice. A: Changes in mesangial expansion, evaluated in HE-stained sections. B: Changes in deposition of fibronectin (FN) in the mesangial spaces. The histological scores are expressed as mean ± S.D. (n=6). An unpaired two-tailed t-test was used to compare the means and a value of *p*<0.05 was considered to have statistical significance. *: *p*<0.05, **: *p*<0.01, as compared to the histological score of age-matched control mice. Typical photographs are shown during the progression of diabetic glomerulosclerosis. See text for abbreviations.
Injuries [15]. In non-diabetic mice, fibronectin was noted along the GBM, but not in the mesangial areas. In contrast, fibronectin-deposited areas were expanded in the glomeruli, especially on the thickened GBM as well as in the mesangial areas from 6 weeks following STZ injection, reaching a peak at 10 weeks after the injection (Fig. 1B). The glomerular fibronectin score gradually increased after the STZ injection, as compared to findings noted in age-matched control mice (10W: diabetic; 1.88 ± 0.32 vs. control; 0.76 ± 0.22, p<0.01) (Fig. 1B). Furthermore, other ECMs (such as type IV collagen and laminin) were evident in the mesangial areas (data not shown), supporting evidence that the present method accelerates glomerular sclerogenesis in mice.

To elucidate the mechanisms by which sclerotic lesions develop, we focused on glomerular vascular components, because cellular events in glomerulosclerosis are similar to those in atherosclerosis [3, 21]. In atherosclerosis, smooth muscle cells show overgrowth in response to a loss (or injury) in endothelial cells and this counterbalance may aggravate vascular sclerotic...
lesions [21]. In our model, the glomerular vonWF staining score decreased in an advanced stage of DN (10W: diabetic; 1.02 ± 0.16 vs. non-diabetic; 1.37 ± 0.22, p<0.05) (Fig. 2A). The reduction in vonWF-positive areas might include over-stretched endothelium. Otherwise, a loss of vonWF may imply a “release” from endothelial cells due to diabetic injuries, as has been suggested elsewhere [6]. Therefore, we counted CD31-positive cells to accurately evaluate endothelial cellularity. As a result, the number of CD-31-positive glomerular cells was significantly fewer in the diabetic mice than age-matched intact mice (Fig. 2B), thereby delineating a decrease in the number of glomerular endothelial cells during the progression of diabetic nephropathy. In contrast, myofibroblastic mesangial cells (as evidenced by α-SMA) increased after STZ-injections (Fig. 2C), which was in agreement with previous reports of human DN patients [4]. During the current observations, there were no significant differences in the number of desmin-positive extra-capillary cells (i.e., podocytes) between diabetic and non-diabetic groups (data not shown), suggesting that diabetic conditions may not alter the cellularity of podocytes in our mouse model. Further studies are needed to determine involvement of podocyte injuries in the diabetic glomerulopathy, as reported elsewhere [24].

We discuss possible mechanisms of glomerular endothelial injuries under hyperglycemia. STZ per se is not toxic toward vascular endothelial cells (unpublished data). On the other hand, high glucose is apoptotic toward vascular endothelial cells [5]. Furthermore, STZ-induced hyperglycemia causes hyperlipidemia and systemic hypertension in vivo [10, 12], while these pathological alterations may worsen endothelial injuries, as has been suggested elsewhere [21]. In human patients, neoangiogenesis occurs in the diabetic kidney around the glomerular vascular poles [14]. One possible explanation for this difference is that human patients undergo supplement therapy with insulin (an angiogenic mediator [13]), while no treatment was performed on the STZ-treated mice. Thus, hyperglycemia may elicit endothelial injuries under “insulin-free” diabetic states. Taken together, sequential processes include: 1) glomerular endothelial injuries (possibly, dysfunction and cell death) occur under hyperglycemia-related conditions; 2) mesangial cells in turn become activated and have a phenotype of myofibroblasts; and 3) finally, ECM is over-accumulated by myofibroblasts, leading to tuft sclerosis. It is well known that advanced glycation end-products are important for provoking diabetic glomerulopathy [25]. In our mouse model, glycation products (such as imidazolone) were deposited in some hypertrophic glomeruli, especially in the nodular tuft areas (data not shown). Therefore, we predict that glomerular glycation deposition may at least in part participate in the onset and progression of diabetic glomerulopathy in our mouse model.

There may be two important factors in the induction of glomerular changes in diabetic mice: one dependent on the genetic background [26], and the other on plasma glucose levels [27]. It has been reported that the C57BL/6 mouse strain is prone to hyperglycemia, atherosclerosis [9] and possibly glomerular changes. With regard to this, Al-Douahji et al. recently described that repeated injections of STZ with a high dose (160 mg/kg/days, i.p., for 2 days) failed to induce glomerular changes within 70 days after STZ injection [1]. This may be due to a difference in intensity of the hyperglycemia: plasma glucose levels in our model reached around 600 mg/dl, whereas those in their model were under 450 mg/dl [1]. Furthermore, they used C57BL/6J mice [1], but this subline is less sensitive to STZ than C57BL/6CrSlc mice (unpublished data).

In summary, we developed a new method to induce DN in C57BL/6 mice, with modifications to the STZ-injection regimen. Early selection of STZ-injected mice based on urine color was helpful in eliciting reproducible outcomes of DN-linked phenotypes (such as renal dysfunction and glomerulopathy). In STZ-treated mice, glomerular changes (including ECM over-deposition and endothelial injuries) are detectable as early as 10 weeks after onset of severe hyperglycemia (plasma glucose >600 mg/dl). Therefore, the onset of sclerosis in our model appeared earlier than has been previously reported [1, 2, 8]. We are now in the process of analyzing the possible involvement of cytokines in DN, using the current STZ protocol.

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References