Roles of Coagulation Pathway and Factor Xa in the Progression of Diabetic Nephropathy in db/db Mice

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The active type of coagulation factor X (factor Xa) activates various cell-types through protease-activated receptor 2 (PAR2). We previously reported that a factor Xa inhibitor could suppress Thy-1 nephritis. Considering that fibrin deposition is observed in diabetic nephropathy as well as in glomerulonephritis, this study examined the roles of the coagulation pathway and factor Xa in the development of diabetic nephropathy using type 2 diabetic model mice. Diabetic (db/db) and normoglycemic (m+/m+) mice were immunohistochemically evaluated for their expression/deposition of PAR2, transforming growth factor (TGF-β), fibrin, extracellular matrix (ECM) proteins, and CD31 at week 20. Significantly greater numbers of PAR2-positive cells and larger amounts of fibronectin, and collagen IV depositions were observed in the glomeruli of db/db mice than those in m+/m+ mice. Next, expression of PAR2 versus deposition of collagen IV and fibronectin was compared between week 20 and week 30, and the number of PAR2-positive cells in the glomeruli decreased in contrast with the increased accumulation of ECM proteins. In an intervention study, fondaparinux, a factor Xa inhibitor, was subcutaneously administered for ten weeks from week 10 to 20. Fondaparinux treatment significantly suppressed urinary protein, glomerular hypertrophy, fibrin deposition, expression of connective tissue growth factor, and ECM proteins deposition together with CD31-positive capillaries. These results suggest that coagulation pathway and glomerular PAR2 expression are upregulated in the early phase of diabetes, together with the increase of profibrotic cytokines expression, ECM proteins deposition and CD-31-positive vessels. Factor Xa inhibition may ameliorate glomerular neoangiogenesis and ECM accumulation in diabetic nephropathy.

Key words diabetes mellitus; factor Xa; nephropathy; collagen IV; transforming growth factor β; protease-activated receptor 2

In various active mesangio proliferative glomerulonephritis, fibrin deposition is often observed in glomeruli, and a pathogenic linkage between locally accelerated coagulation and mesangial proliferation is suspected. Also in human and experimental diabetic nephropathy, glomerular fibrin deposition and extracellular matrix (ECM) expansion have been noted. In this context, transforming growth factor (TGF)-β is upregulated in kidney diseases, and TGF-β promotes the production of various ECM proteins. Regarding its relationship with coagulation, thrombin stimulates TGF-β production in mesangial cells in vitro. On the other hand, plasminogen-activator-inhibitor type 1 (PAI-1) is an inhibitor of the fibrinolytic system and is known to increase ECM components through the inhibition of plasmin. In diabetes mellitus, impaired fibrinolytic activity may lead to vascular abnormalities and fibrosis, and patients with greater urinary albumin excretion exhibit a significantly higher ratio of PAI-1/tissue-type plasminogen activator in the circulation. It is generally accepted that ECM accumulation in diabetic kidney progresses to nephrosclerosis, and as a consequence diabetic nephropathy has become a major cause of end-stage renal failure.

Tissue factor is known to be the initiator of the extrinsic coagulation cascade that converts factor X to its active form, Xa. Factor V in its active form (Va) serves as a membrane-bound cofactor to factor Xa, forming a factor Xa/Va complex, which promotes activation of prothrombin to thrombin. Then, thrombin immediately converts fibrinogen to fibrin monomers, and finally factor XIIIa induces polymerization of cross-linked fibrin from the fibrin monomers. On the other hand, thrombin and factor Xa activate protease-activated receptors (PARs), a novel family of G protein-coupled cell-surface receptors.

We previously reported that the coagulation process proceeds together with the ECM accumulation through factor V expression in rat Thy-1 nephritis, and that DX-9065a, a factor Xa inhibitor, suppresses this type of glomerulonephritis. Because renal tissue factor expression and fibrin deposition are observed in streptozotocin-induced type 1 diabetic mice, it is suspected that the coagulation process is activated through factor Xa formation in diabetic nephropathy. It has been reported that fondaparinux, a synthetic pentasaccharide that selectively inhibits factor Xa, reduces the inflammatory response in injured kidneys due to ischemia-reperfusion. The aim of the present study, therefore, was to clarify the contributions of the coagulation system and factor Xa on ECM accumulation and angiogenesis in diabetic nephropathy. The results revealed an important role for coagulation pathway in glomerular hypertrophy and ECM accumulation of diabetic nephropathy and the protective mechanisms of factor Xa regulation in this disorder.

MATERIALS AND METHODS

Materials Nine-week-old male db/db mice (C57BLKS/J-db/db) weighing 35 to 40 g were obtained from Japan CLEA (Tokyo, Japan). This strain is obese and known to develop
type 2 diabetes, followed by diabetic kidney disease.\(^{20}\) As a control group, age-matched non-diabetic m+/m+ littersmates weighing 18 to 23 g were also purchased from the same company. Blood glucose measurements obtained with a glucometer (Glucose Pilot; Iwai Chemicals, Tokyo, Japan) confirmed that the m+/m+ mice were normoglycemic (at the age of 9 weeks old, 148±18 mg/dl; 19 weeks old, 152±9 mg/dl; 30 weeks old, 134±5 mg/dl), and the db/db mice were hyperglycemic (at the age of 9 weeks old, 429±55 mg/dl; 19 weeks old, over 600 mg/dl; 30 weeks old, 522±78 mg/dl). Animal care and experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka, and the Review Board of the University granted ethical permission for this study. Fondaparinux, a synthetic pentasaccharide with molecular weight 1728, was kindly provided by GlaxoSmithKline (Brentford, Middlesex, U.K.).

**Experimental Design** After purchase, mice were maintained to week 20 with free access to food and water until they were sacrificed, and their kidneys were collected (n=6 in each group). Kidney tissues were stained for ordinary light microscopy and immunohistolological evaluation, as described below. For the time course study of PAR2 expression and collagen IV deposition, db/db mice were killed at weeks 20 and 30 (n=6 in each group), and the histology was compared between the groups.

Then, for the intervention study, 6 db/db mice were divided into two experimental groups (n=3 in each group): the disease control group and the fondaparinux treatment group. Fondaparinux was subcutaneously administered at a dose of 0.5 mg/kg in 0.2 to 0.25 ml of saline three times/week according to body weight gains from week 10 to 20. In the control group, the same volume of physiological saline was subcutaneously injected with the same intervals. All mice were sacrificed at week 20, when their kidneys were collected. Before treatment and at the time of sacrifice, 24-h urine collections for measuring creatinine and protein levels were obtained from each mouse. The prothrombin time was assessed.

**Measurement of Blood Pressure (BP), Urinary Protein, and Serum Creatinine** Systolic BP was measured in the conscious state by tail cuff method (Softron, Tokyo, Japan). To measure urinary albumin excretion, a Mouse Albumin ELISA Quantitation kit (Bethyl Laboratories, Montgomery, TX, U.S.A.) was used according to the manufacturer’s protocol. Horseradish peroxidase-labeled goat antibody against mouse albumin was detected with 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium (Wako Pure Chemical Industries, Osaka, Japan) at an absorbance of 405 nm. Urine volume was markedly different between normoglycemic m+/m+ and diabetic db/db mice. Therefore, we compared albumin/creatinine instead of albumin excretion. Serum and urine creatinine levels were determined by an enzymatic method (CRE-EN, KAINOS Laboratories, Tokyo, Japan).

**Histological Evaluation and Immunoperoxidase Staining for Light Microscopy** Kidney tissues from each animal were processed for evaluation by light microscopy and immunostaining microscopy. For light microscopy, the tissues were fixed in 10% neutral-buffered formalin (pH 7.4) and embedded in paraffin. Then, sections (4 µm) were subjected to periodic acid-Schiff staining, which was evaluated quantitatively by measuring the size of the glomerular area in 10 randomly selected glomerular cross sections.

Tissue specimens frozen in OCT compound (Miles Laboratories, Elkhart, IN, U.S.A.), were cut into serial sections on a cryostat, and fixed in acetone for 5 min at room temperature. The detection of PAR2, factor X, TGF-β, connective tissue growth factor (CTGF), fibrin, fibrinectin, and collagen IV was performed by an indirect method using a goat anti-mouse PAR2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), a goat anti-human factor X antibody (Santa Cruz Biotechnology), a goat anti-human TGF-β antibody (Santa Cruz Biotechnology), a goat anti-human CTGF antibody (GT, Minneapolis, MN, U.S.A.), a goat antibody against mouse fibrinogen (Nordic Immunological Laboratories, Tilburg, the Netherlands), a goat antibody against rat fibronectin (Santa Cruz Biotechnology), and a rabbit antibody against mouse type IV collagen (Santa Cruz Biotechnology), as previously described.\(^{16}\) Briefly, sections were incubated with each primary antibody for 1 h, followed by biotinylated immunoglobulin G (IgG) secondary antibody (Vector Laboratories, Burlingame, CA, U.S.A.). The sections were then reacted with avidin–D–biotinylated horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories). The color was then developed by incubation with a DAB Substrate kit (Pierce, Rockford, IL, U.S.A.) and the sections were counterstained with hematoxylin.

Other deparaffinized sections underwent microwave antigen retrieval and were then incubated with a goat anti-mouse platelet-endothelial cell adhesion molecule-1 antibody (PECAM, CD31) (Santa Cruz Biotechnology) for 1 h at room temperature followed by over/night incubation at 4°C. Next, the sections were incubated with a secondary antibody, and the color was developed as mentioned above. The average number of CD-31 positive glomerular capillaries was determined by observing 10 glomeruli in each section, as described previously.\(^{21}\)

The average number of PAR2-positive cells in a glomerular cross section was evaluated by counting the cells in 10 glomeruli in each section. The glomerular positive-staining areas of TGF-β, fibrin, fibrinectin, and collagen IV were evaluated quantitatively in 10 selected glomerular cross sections by NIH Image version 1.62 (NIH, Bethesda, MD, U.S.A.) and were expressed as percentage of the total staining area of the glomeruli.

**Western Immunoblot Analysis** For protein extracts preparation, renal cortex tissues were homogenized in lysis buffer with a Polytron (Kinematics AG, Cincinnati, OH, U.S.A.). The protein extracts were put on ice for 30 min and spun at 12000 rpm. The supernatant was then collected. Proteins were separated by sodium dodecyl sulfate (15.0%) polyacrylamide gel electrophoresis using 25 µg of protein per sample. Resultant proteins were electrobotted onto polyvinylidene fluoride membranes. Membranes were then incubated for 3 h at 37°C in 5% skim milk solution. The resultant blots were incubated at room temperature for 2 h with each of a rabbit anti-human TGF-β (Santa Cruz Biotechnology) or a rabbit anti-mouse β-actin (AmaSpec, San Jose, CA, U.S.A.). Blots were washed and incubated with a rabbit anti-goat IgG-horse radish peroxidase (HRP). The antigen–antibody complexes were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica,
Statistical Analysis Values are represented as means±S.E.M. Statistical differences were assessed using the Kruskal–Wallis tests followed by Mann–Whitney tests. Differences at p<0.05 were considered statistically significant.

RESULTS

Urinary Findings and Blood Pressure The amount of urinary albumin was markedly increased in the db/db group compared to the m+/m+ group (Table 1). Although in the m+/m+ group the mean albumin excretion was less than 40 mg/mg creatinine during weeks 9 to 26, in the db/db group, it was massive and over 800 mg/mg creatinine for the same period. Systolic blood pressure did not show any significant difference between m+/m+ and db/db mice groups at week 20; m+/m+, 125±4 (n=4); and db/db 122±5 mmHg (n=6).

Immunostaining for PAR2, factor X, TGF-β PAR2, factor X, and TGF-β were immunohistochemically detected in the db/db mice at week 20, and representative findings for m+/m+ and db/db mice are shown in Fig. 1. Although PAR2-positive cells were scarcely observed in the mice of m+/m+ group, such positive cells were abundantly noted in the glomeruli of db/db group (Figs. 1A, D). Factor X staining was scarce in the m+/m+ group, and was obvious in the db/db group (Figs. 1B, E). TGF-β staining in the glomeruli of db/db group was increased, compared with that in the m+/m+ group (Figs. 1C, F).

Time Course of the Expression of PAR2 and Collagen

<table>
<thead>
<tr>
<th>Week</th>
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<tr>
<td>9</td>
<td>25±5</td>
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<tr>
<td>12</td>
<td>29±9</td>
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<td>20</td>
<td>25±7</td>
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<td>26</td>
<td>29±4</td>
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<tr>
<th>Mice group</th>
<th>Urinary Findings and Blood Pressure</th>
<th>Week 20</th>
<th>Week 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>m+/m+</td>
<td>125±4 (n=4)</td>
<td>122±5  (n=6)</td>
<td></td>
</tr>
<tr>
<td>db/db</td>
<td>122±5  (n=6)</td>
<td>125±4  (n=6)</td>
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IV Accumulation In our preliminary study, PAR2 expression was scarce in the human diabetic glomeruli of advanced stage (data not shown). In this study, to find out whether PAR2 expression increases along with the progression of diabetic sclerosis, we examined PAR2 expression together with the accumulation of collagen IV and fibronectin in both m+/m+ and db/db mice at weeks 20 and 30. The number of PAR2-positive cells per glomerulus (A), percent of collagen IV (B), and percent of fibronectin-staining area per total glomerular area (C) were shown. The glomeruli of the normoglycemic m+/m+ (open column) and diabetic db/db group (closed column) are shown. *p<0.05 vs. m+/m+ mice at the same age; and #p<0.05 vs. db/db mice at week 20.

<table>
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<th>Week 30</th>
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<td>122±5  (n=6)</td>
<td></td>
</tr>
<tr>
<td>db/db</td>
<td>122±5  (n=6)</td>
<td>125±4  (n=6)</td>
<td></td>
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Table 1. Comparison of Urinary Albumin Excretion between m+/m+ and db/db Mice

Table 2. Comparison of PAR2 Expression, Deposition of Collagen IV, and Fibronectin between m+/m+ and db/db Mice

<table>
<thead>
<tr>
<th>Week</th>
<th>PAR2 expression positive cells/glomerulus</th>
<th>Deposition of collagen IV % of glomerular area</th>
<th>Deposition of fibronectin % of glomerular area</th>
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<tbody>
<tr>
<td>20</td>
<td>3.2±0.4</td>
<td>27.5±0.9</td>
<td>14.6±0.6</td>
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<tr>
<td>30</td>
<td>9.9±0.5*</td>
<td>32.4±1.2*</td>
<td>26.9±1.5*</td>
</tr>
</tbody>
</table>

* p<0.05 versus m+/m+ mice at the same age; and #p<0.05 versus db/db mice at week 20.

Fig. 1. Immunohistological Findings of PAR2, Factor X, and TGF-β in the Glomeruli of m+/m+ and db/db Mice

PAR2 (A, D), factor X (B, E), and TGF-β (C, F) were stained in renal tissues. The glomeruli of the normoglycemic m+/m+ (A—C) and diabetic db/db group (D—F) are shown. Original magnification, ×400.

Fig. 2. Quantitative Comparison of Immunohistochemical Findings between m+/m+ and db/db Mice

Number of PAR2-positive cells per glomerulus (A), percent of collagen IV (B), and percent of fibronectin-staining area per total glomerular area (C) were shown. The glomeruli of the normoglycemic m+/m+ (open column) and diabetic db/db group (closed column) are shown. *p<0.05 versus m+/m+ mice at the same age; and #p<0.05 versus db/db mice at week 20 (6 mice per group).
PAR2-positive cells in the glomeruli of the db/db group was significantly higher than the number of the m+/m+ group (Table 2, Fig. 2A). PAR2-positive cells were abundantly observed with moderate deposition of collagen IV (Table 2, Fig. 2B) and fibronectin (Table 2, Fig. 2C) at week 20. Interestingly at week 30, although the accumulation of collagen IV and fibronectin was advanced in the db/db mice, the number of PAR2-positive cells in db/db mice was decreased compared with that at week 20.

The Effect of Fondaparinux on Body Weight, Prothrombin Time, Proteinuria, Blood Glucose, Serum Creatinine Levels, and Lipid Profiles When mice were sacrificed at week 20 after intervention study for 10 weeks, body weight markedly increased in the db/db control group than in the m+/m+ group, and the fondaparinux treatment did not influence significantly (Table 3). The prothrombin time was not significantly changed. The amount of urinary albumin was markedly increased in the db/db control group than in the m+/m+ group, and the fondaparinux treatment significantly reduced urinary protein (Fig. 3). Blood glucose was markedly increased in the db/db control group than in the m+/m+ group, and the fondaparinux treatment did not change blood glucose levels. Serum creatinine levels were not significantly changed among groups. Total cholesterol was markedly increased in the db/db control group than in the m+/m+ group, and the fondaparinux treatment did not change the levels; and triglyceride was not significantly changed. Serum creatinine levels were not significantly changed among groups. Total cholesterol was significantly suppressed by the fondaparinux treatment (Table 3).

Table 3. Comparison of Body Weight, Prothrombin Time, Proteinuria, Blood Glucose, Serum Creatinine Levels, and Lipid Profiles before and after Fondaparinux Treatment

<table>
<thead>
<tr>
<th></th>
<th>m+/m+</th>
<th>Control</th>
<th>Fondaparinux treatment</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>27.4±0.7</td>
<td>48.7±8.0*</td>
<td>48.7±0.2</td>
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<tr>
<td>Prothrombin time (s)</td>
<td>19.1±0.2</td>
<td>18.5±0.5</td>
<td>17.7±0.0</td>
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<tr>
<td>Urinary albumin (mg/mg creatinine)</td>
<td>0.04±0.0</td>
<td>2.02±0.01*</td>
<td>1.68±0.11*</td>
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<tr>
<td>Blood glucose (mg/dl)</td>
<td>171±17</td>
<td>594±6*</td>
<td>570±25</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>1.33±0.02</td>
<td>1.37±0.03</td>
<td>1.30±0.04</td>
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<tr>
<td>Total cholesterol (mg/dl)</td>
<td>63±8</td>
<td>112±13*</td>
<td>113±34</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>59±3</td>
<td>140±48</td>
<td>97±42</td>
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</table>

* p<0.05 vs. m+/m+ mice; and # p<0.05 vs. control db/db.

The Effects of Fondaparinux on Glomerular Size We next examined the glomerular histological change in these mice after fondaparinux treatment. The glomerular area was 60% greater in the db/db control than in the m+/m+ group at week 20 (p<0.05). The glomerular hypertrophy was markedly reduced by fondaparinux treatment (p<0.05 vs. db/db control) (Table 4).

Table 4. Comparison of Glomerular Size, CTGF Expression, Fibrin Deposition, Collagen IV Accumulation, and Factor X Deposition

<table>
<thead>
<tr>
<th></th>
<th>m+/m+</th>
<th>Control</th>
<th>Fondaparinux treatment</th>
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<td></td>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=3)</td>
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<tr>
<td>Glomerular size ×10³ μm²</td>
<td>1.68±0.12</td>
<td>2.70±0.10*</td>
<td>2.22±0.04*</td>
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<tr>
<td>CTGF expression % of m+/m+</td>
<td>100.0±32.9</td>
<td>236.4±21.0*</td>
<td>155.3±25.6*</td>
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<tr>
<td>Fibrin deposition % of m+/m+</td>
<td>100.0±2.3</td>
<td>293.8±12.5*</td>
<td>209.5±27.8*</td>
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<tr>
<td>Collagen IV accumulation % of m+/m+</td>
<td>100.0±0.4</td>
<td>161.1±6.8*</td>
<td>113.5±7.1*</td>
</tr>
<tr>
<td>TGF-β by immunoblotting ratio to β-actin</td>
<td>0.16±0.02</td>
<td>0.60±0.08*</td>
<td>0.48±0.03</td>
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<tr>
<td>CD31-positive capillaries number</td>
<td>9.6±1.6</td>
<td>22.9±2.2*</td>
<td>17.1±1.8*</td>
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</table>

* p<0.05 vs. m+/m+ mice; and # p<0.05 vs. control db/db.

Fig. 3. Suppressive Effects of Fondaparinux on Urinary Protein

Levels of urinary albumin excretion are adjusted by urinary creatinine excretion. The amounts of urinary protein in the control db/db group were significantly higher than those in the m+/m+ group, but reduced in the fondaparinux-treated group. ∗ p<0.05 vs. m+/m+ group; and # p<0.05 vs. control db/db group (3 mice per group).

Fig. 4. Suppressive Effects of Fondaparinux on Glomerular Size

Glomerular hypertrophy is observed in control db/db group (B) and fondaparinux-treated group (C), compared with the normal group (A). Representative photos are shown. After the quantitative analysis, it was revealed that glomerular hypertrophy was significantly suppressed by the fondaparinux treatment (D). ∗ p<0.05 vs. m+/m+ group; and # p<0.05 vs. control db/db group (3 mice per group). Original magnification, ×400.
db/db control than in the m+/m+ group were observed ($p<0.05$, respectively), and suppression of fibrin and collagen IV deposition was also noted ($p<0.05$ vs. db/db control, respectively) (Figs. 5K, L). On the other hand, factor X deposition did not differ between db/db control group and fondaparinux-treated db/db group (data not shown).

**DISCUSSION**

In this study, we found 1) abundant PAR2-positive cells, 2) increased amounts of TGF-β, fibrin-related antigen, fibronectin, and collagen IV depositions, 3) and neoangiogenesis in the glomeruli of diabetic db/db mice. Interestingly, the increase in the number of PAR2-positive cells was blunted by the progression of collagen IV and fibronectin deposition through aging. We also found that fondaparinux, a specific factor Xa inhibitor, could ameliorate the characteristic features of diabetic nephropathy (proteinuria, glomerular hypertrophy, ECM accumulation, fibrin deposition, and angiogenesis), suggesting roles of coagulation pathway and factor Xa.
in the fibrogenesis of diabetic nephropathy.

One of the characteristics of anti-Xa treatment as anticoagulants, it is pointed that hemorrhagic adverse event is low. 22) Compared with the clinical dose of fondaparinux treatment is subcutaneous 0.05 mg/kg per day, our dosage in this study is 5 times larger: subcutaneous 0.5 mg/kg for three times per week (nearly 0.25 mg/d). As a consequence glomerular fibrin deposition was decreased in the fondaparinux-treatment group, compared with the non-treated db/db mice. Although Frank et al. reported that single administration of fondaparinux attenuated plasma Xa activity for 4 h after the intra-venous injection of 5 mg/kg dose in normal mice, and that treatment with 7.5 mg/kg of fondaparinux just before declamping and 5 mg/kg after declamping in ischemia/reperfusion injury mice induced prolonged anti-factor Xa activity at 24 and 48 h, 23) we observed not particular prolongation of plasma prothrombin time after the continuous subcutaneous administration of fondaparinux for ten weeks. This could be due to the gradual absorption route of intra-subcutaneous injection and the low dose compared with the study design of Frank et al., and our dose and administration design may be near to the clinical usage.

It is accepted that the renin–angiotensin–aldosterone (RAA) system is a key mediator in diabetic nephropathy, and that inhibitors of this system, such as angiotensin-converting enzyme inhibitors and angiotensin type 1 receptor blockers, prevent this type of nephropathy from progressing to end-stage renal failure. 23,24) On the other hand, heparin therapy is known to have a proteinuria-lowering effect, which cannot be explained via the RAA system. 25) van der Pijl et al. reported that danaparoid, which is a mixture of sulfated glycosaminoglycans, significantly lowered proteinuria in type 1 diabetic nephropathy. 26) They suggested that danaparoid supplements the action of proteoglycans, rather than inhibiting factor Xa. Indeed, heparin is known to have various effects other than anticoagulation. For example, hepatocyte growth factor has anti-fibrotic effects and its plasma levels increased after heparin treatment. 27) Furthermore, non-anticoagulant heparin is also effective at ameliorating Thy-1 nephritis. 28) Therefore, we were interested in the efficacy of fondaparinux, a specific factor Xa inhibitor, against diabetic nephropathy in db/db mice.

Previously Farquhar et al. reported that 70% of glomeruli showed fibrin-related antigens along the glomerular capillary wall and diffusely in the mesangium, although biopsy specimens showed only early to moderate stage diabetic nephropathy. 29) They concluded that endothelial and mesangial trapping of fibrinogen and/or other macromolecules might initiate or accelerate mesangial enlargement and irregularities of the basement membrane. In glomerulonephritis, intraglomerular coagulation with fibrin deposition was also suggested to be involved in the development of glomerular injury. 29) Among various coagulation factors, tissue factor is known to be the principle initiator of the extrinsic coagulation pathway, and the glomerular expression of tissue factor is upregulated in human and rabbit crescentic glomerulonephritis. 30,31) as well as in experimental diabetic nephropathy. 38) Tissue factor and factor V are produced in mesangial cells after inflammatory stimulation. 32,33) Factor Xa is formed after the activation of factor X. If factor X is increased together with fibrin, one can assume that factor Xa is also increased. Because fondaparinux treatment significantly reduced glomerular fibrin deposition and did not significantly reduced factor X deposition in this study, it is suspected that fondaparinux performed suppression on factor Xa activity, rather than reduction of factor X quantity. Factor Xa is not only a key factor in coagulation cascades, but also a potent mitogen for endothelial cells. 34) Furthermore, thrombin stimulates TGF-β production in cultured mesangial cells, 35) and factor Xa upregulates the synthesis of TGF-β in cultured tu-
Thrombin and factor Xa activate the PAR family. After selective cleavage of the receptors by these serine-proteases, PARs mediate signaling, and thereby exert various cellular effects that induce inflammatory responses to tissue injury. Several in vitro studies have reported that PAR2 can mediate factor Xa signaling, but not thrombin signaling. PAR2 is expressed in cultured human mesangial cells, and factor Xa induces cellular proliferation through the activation of extracellular regulated kinase (ERK) via PAR2, as demonstrated previously by our group. Yamamoto et al. reported that tumstatin peptide, an inhibitor of angiogenesis, prevented glomerular hypertrophy in the early stage of streptozotocin-induced diabetic nephropathy. Recently, Uusitalo-Jarvinen et al. have shown using genetically deficient mice that PAR1 and PAR2 play roles in hypoxia-driven angiogenesis and that PAR2 signaling is sufficient for this proangiogenic effect.

In the present study, the factor Xa inhibitor fondaparinux suppressed both glomerular hypertrophy and hypervascularity in db/db mice. Taken together, it is tempting to speculate that fondaparinux inhibits glomerular hypertrophy by the suppressing angiogenesis through PAR2 regulation. It is known that PAR2 expression in cultured fibroblasts is suppressed within the collagen matrix. Consistent with this, we found that the increases observed in the number of PAR2-positive cells of db/db mice at week 20 were reduced by the progression of collagen IV and fibronectin deposition through aging at week 30. Therefore, PAR2 expression might be self-limited by the production of ECM proteins, surround mesangial cells due to PAR2 activation. Because diabetic nephropathy progresses to end-stage renal failure due to ECM accumulation, it is suspected that regulation of PAR2 activation in the early phase of nephropathy, using a factor Xa inhibitor, would be a useful method.

In conclusion, it is suspected that coagulation pathway and glomerular PAR2 expression are upregulated in the early phase of diabetes, together with the increase of profibrotic cytokines expression, extracellular matrix (ECM) proteins deposition and CD-31-positive vessels. Factor Xa inhibition may ameliorate glomerular neoangiogenesis and ECM accumulation in diabetic nephropathy.

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Conflict of Interest Statement None declared.

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