The Lack of Cyclin Kinase Inhibitor p27\textsuperscript{Kip1} Ameliorates Progression of Diabetic Nephropathy

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Abstract. Cyclin kinase inhibitor p27\textsuperscript{Kip1} (p27) has been shown to be upregulated in glomeruli of diabetic animals and mesangial cells cultured under high glucose. This study was an investigation of the role of p27 in the progression of diabetic nephropathy. Mice deficient in p27 (p27\textsuperscript{−/−}) and wild-type mice (p27\textsuperscript{+/+}) were studied 12 wk after diabetes induction by streptozotocin. Blood glucose and BP were comparable between diabetic p27\textsuperscript{+/+} and p27\textsuperscript{−/−} mice. The kidney weight to body weight ratio and glomerular volume increase in diabetic p27\textsuperscript{+/+} mice. In contrast, these parameters did not change in diabetic p27\textsuperscript{−/−} mice. Similarly, albuminuria developed in diabetic p27\textsuperscript{+/+} mice but not in diabetic p27\textsuperscript{−/−} mice. The mesangial expansion was significantly milder in diabetic p27\textsuperscript{−/−} mice than that in diabetic p27\textsuperscript{+/+} mice. These changes were associated with a similar increase in glomerular TGF-β expression in diabetic p27\textsuperscript{+/+} and p27\textsuperscript{−/−} mice. However, glomerular protein expression of fibronectin, a target of TGF-β, increased only in diabetic p27\textsuperscript{+/+} mice. In mesangial cells cultured from p27\textsuperscript{+/+} mice, exposure to high glucose caused significant increases in total protein content and [\(^{3}H\)]-leucine incorporation. On the other hand, high glucose caused a significant reduction in these parameters in cells from p27\textsuperscript{−/−} mice. Phosphorylation of 4E-BP1, the translation inhibitor, increased after exposure to high glucose in p27\textsuperscript{+/+} cells. In p27\textsuperscript{−/−} cells, the level of phosphorylated 4E-BP1 was higher than that in control p27\textsuperscript{+/+} cells and decreased under high glucose conditions. In conclusion, renal hypertrophy, glomerular hypertrophy, and albuminuria did not develop, and mesangial expansion was milder in diabetic p27\textsuperscript{−/−} mice despite glomerular TGF-β upregulation. These results suggest that controlling p27 function may ameliorate diabetic nephropathy.

Early diabetic nephropathy is characterized by hypertrophy of both glomerular and tubuloepithelial elements (1). Cellular hypertrophy is associated with increased synthesis and accumulation of extracellular matrix, which ultimately result in glomerulosclerosis and tubulointerstitial fibrosis (2). Transforming growth factor-β (TGF-β) is considered to play a pivotal role in these processes. Recently, the mechanisms of hypertrophy have been elucidated at the cellular and molecular level (3,4). Thus, cells undergo hypertrophy when they arrest at the late G1 phase failing to progress into the S phase. Cell cycle is controlled by multiple cell cycle regulatory proteins. Progression through the cell cycle requires the activation of cyclin-dependent kinases (CDK) by cyclins (5). The G1-phase cyclins D and E activate CDK4/6 and CDK2, respectively, phosphorylating the retinoblastoma protein. The activity of CDK is modulated by cyclin kinase inhibitors (CKI). There are two distinct families of CKI, i.e., the p21\textsuperscript{Cip1} (p21)/p27\textsuperscript{Kip1} (p27) family and the p15\textsuperscript{Ink4B}/p16\textsuperscript{Ink4A} family. While p21 and p27 inhibit almost all cyclin-CDK complexes, p16 interacts only with CDK4 and CDK6. Of these, p21 and p27 have been shown to be responsible for hypertrophy in renal tubular cells (6). Thus, overexpression of p21 or p27 caused hypertrophy in LLC-PK\(_1\) cells, but p16 had no such effect. Overexpression of p16 inhibited CDK4 activity but did not affect CDK2 activity, whereas overexpression of p21 or p27 inhibited both. It was postulated that arrest at the late G1 phase was necessary for the induction of hypertrophy.

The importance of p21 in mesangial cell and glomerular hypertrophy in experimental diabetes has been suggested by Kuan et al (7). Both high glucose-induced mesangial cell hypertrophy and glomerular hypertrophy in streptozotocin-induced diabetic mice were accompanied by elevated levels of p21. Investigators from the same laboratory further demonstrated that glomerular hypertrophy did not develop in diabetic mice lacking p21 despite an increase in TGF-β expression, indicating the importance of p21 in diabetic nephropathy (8).

The critical role of p27 in cellular hypertrophy induced by angiotensin II or high glucose has been demonstrated in a variety of cell types. Wolf and Stahl (9) showed that the expression of p27 was upregulated in proximal tubular cells stimulated by angiotensin II and that p27 antisense oligonucleotide abolished the angiotensin II-mediated hypertrophy. Braun-Dullaeus et al. (10) also demonstrated that p27 played an important role in mediating angiotensin II-stimulated vascular smooth muscle cell hypertrophy. Furthermore, upregulation of p27 was associated with mesangial cell hypertrophy.
under high ambient glucose (11). Again, p27 antisense oligonucleotide abolished high glucose-stimulated protein synthesis and G1 phase arrest. More recently, Wolf et al. (12) reported that mesangial cells isolated from p27−/− mice did not undergo hypertrophy under high glucose conditions despite an increase in TGF-β. Reconstituting p27 by transient or stable transfection increased protein synthesis and restored G1 phase arrest.

While increased expression of p27 in mesangial cells and podocytes has been demonstrated in diabetic db/db mice (13), in vivo significance of p27 in diabetes has not been investigated so far. In the present study, therefore, we examined the role of p27 in the progression of diabetic nephropathy using mice lacking p27.

Materials and Methods

Materials

Streptozotocin (STZ) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Monoclonal anti-TGF-β antibody was from Genzyme (Cambridge, MA). A monoclonal antibody specific for proliferating cell nuclear antigen (PCNA) and cyclin and peroxidase-conjugated rabbit anti-mouse Ig were from DAKO A/S (Glostrup, Denmark). Anti-mouse fibronectin antibody was from Biomedical Technologies (Stoughton, MA). Rabbit polyclonal phospho-4E-BP1 (Thr70) antibody was from New England Biolabs (Beverly, MA). Rabbit polyclonal anti-4E-BP1 (R-1133) was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-α-tubulin, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and N,N′-dimethylformamide were from Sigma (St. Louis, MO).

Animal Model

Mice heterozygous for the p27 gene with C57BL/6 genetic background were kind gift from Nippon Roche Research Center (14). Mice deficient in p27 (p27−/−) and control wild-type mice (p27+/+) were selected from the offspring of heterozygous matings. The genotype was determined by PCR analysis on DNA isolated from tail biopsies as reported previously (14).

Induction of Diabetes

Diabetes was induced in adult 23 to 35 g mice with two consecutive daily intraperitoneal injections of STZ (200 mg/kg) dissolved in 10 mM Na citrate, pH 5.5. Mice matched for age, sex, and weight at the time of STZ administration served as controls (aged 16 to 24 wk, two female and three male pairs for short-term diabetes and three female and three male pairs for long-term diabetes). The mice were sacrificed 3 wk or 12 wk after the day that glucosuria was first present. Mice were decapitated and blood was obtained for determination of non-fasting plasma glucose by the glucose oxidase method. Before sacrificing mice with long-term diabetes, BP was measured by tail cuff method using BP-98A (Softron, Japan). Individual mice were then placed in metabolic cages to obtain 24-h urine collections. Urine creatinine and albumin were measured by an alkaline picate method and an enzyme-linked immunosorbent assay, respectively.

Estimation of Glomerular Volume and Mesangial Area

Kidneys were fixed with neutral buffered formalin. Then 3-μm-thick sections were cut and stained with hematoxylin and eosin. Mean glomerular tuft volume (GV) was determined from the mean glomerular cross-sectional area (GA) by light microscopy as described previously (15). Photomicrographs were scanned, and profile areas were traced using NIH IMAGE. GA was determined as the average area of a total of 30 cortical glomeruli. GV was calculated as

$$GV = \beta/k \times (GA)^{3/2}$$

where $\beta = 1.38$, which is the shape coefficient for spheres (the idealized shape of glomeruli), and $k = 1.1$, which is a size distribution coefficient. To quantify mesangial expansion, 20 glomeruli cut at their vascular pole were used in each animal. Mesangial area was defined as PAS-positive and nuclei-free area in mesangium.

Immunohistochemistry

After fixation with neutral buffered formalin, kidneys were embedded in paraffin. Immunohistochemical staining was performed on serial sections 3-μm-thick, using enzyme-labeled antibody method. Paraffin sections were deparaffinized and rehydrated. Endogenous peroxides activity was quenched by incubating sections in 0.3% H2O2/methanol for 15 min. To unmask antigens, slides were boiled 100°C for 10 min in 10% citrate buffer (pH 6.0)/methanol. Sections were incubated with antibodies against PCNA (dilution 1:100). The incubation time was 60 min at room temperature or overnight at 4°C. After incubating with secondary antibody at a concentration of 1:100, immunoreaction products were developed using 3,3′-diaminobenzidine as the chromogen, with standardized development times. Sections were then counterstained with methyl green.

Histologic Assessments

Quantitation of nuclear staining for PCNA from glomeruli and tubulointerstitium was performed on individual mice in a blinded fashion. At least 20 sequentially selected fields of renal cortex or glomeruli at ×400 magnification were examined.

Isolation of Glomeruli

Glomeruli were isolated from mouse kidneys using a sieving method. The sieved specimens were then collected, centrifuged 3 min × 2000 rpm, and supernatant was discarded. This washing was repeated two more times, and glomeruli were suspended in lysis buffer containing 20 mM Hepes, pH 7.2, 1% Triton-100, 10% glycerol, 20 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin. Glomeruli were homogenized by Dounce, and insoluble material was removed by centrifugation (10,500 × g; 10 min). Protein content in cell lysates was determined by DC protein assay (Bio-Rad Laboratories, Tokyo, Japan).

Immunoblot Analyses

Cleared lysates were resolved by SDS-PAGE and transferred to PVDF membranes (Immobilon, Millipore Corp, Bedford, MA). Non-specific binding sites were blocked in TBS buffer (10 mM Tris-CI, pH 7.4, 0.15 M NaCl) containing 5% skim milk overnight at 4°C or for 1 h at 25°C. Antibodies were added to TBS and incubated with mixing for 2 h at 25°C. Blots were washed and further incubated with a peroxidase-conjugated secondary antibody for 1 h with mixing and washed. Bound antibodies were detected using the ECL Western blotting system from Amersham (Tokyo, Japan). Alternatively, membranes were developed by the addition of 20 mg of nitroblue tetrazolium in 60 ml of substrate buffer (0.1 M Tris-CI, pH 9.5, 0.1 M NaCl, and 5 mM MgCl2) mixed immediately before blot exposure with 10 mg of 5-bromo-4-chloro-3-indolyl phosphate in 200 μl of N,N′-dimethylformamide.
Mesangial Cell Culture

Mouse mesangial cells were isolated and cultured using the method described elsewhere (16). Cells were grown in regular RPMI 1640 containing 17% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 μg/L fungizone. The cultures were maintained at 37°C in a humidified atmosphere of 95% O₂/5% CO₂. Cells were grown in normal glucose RPMI 1640 (NG, glucose 5.5 mM) or high glucose medium (HG, glucose 30 mM) containing 1% FBS.

Measurement of Cell Protein and Cell Number

Cells were subcultured in a 24-well dish at a density of 1 × 10⁴ ml. After 24 h, cells were treated with NG or HG medium with daily exchange. After 5 d, cells were washed with ice-cold PBS and trypsinized for counting cell number, solubilized in 0.5 N NaOH for protein content determination, or lysed for immunoblot analysis. Cell number was counted using a hemocytometer.

[^3H]-Leucine and[^3H]-Thymidine Incorporation

Cells were cultured in a 24-well dish under NG or HG. After 4 d, cells were pulsed with 1 μCi [³H]-leucine or [³H]-thymidine. After incubation for 24 h, cells were washed with ice-cold PBS and 5% TCA, solubilized in 0.5 N NaOH, and counted by a liquid scintillation counter.

Statistical Analyses

The results are expressed as mean ± SEM. Statistical analyses were performed with unpaired t test or ANOVA followed by multiple comparisons as appropriate. Statistical significance was determined as P < 0.05.

Results

Animal Data

Diabetic p27 +/+ mice and diabetic p27 −/− mice exhibited a similar degree of hyperglycemia at 3 wk (Table 1). Body weight, kidney weight, and kidney weight to body weight ratio were numerically or significantly greater in control p27 −/− mice than those in control p27 +/+ mice. Kidney weight corrected for body weight was significantly greater in both diabetic p27 +/+ and p27 −/− mice compared with their controls.

The mean plasma glucose of diabetic p27 +/+ mice and diabetic p27 −/− mice at 12 wk was also comparably elevated (Table 2). There was no difference in BP between p27 +/+ mice and p27 −/− mice. Body weight, kidney weight, and kidney weight to body weight ratio tended to be greater in control p27 −/− mice than those in control p27 +/+ mice. Kidney weight to body weight ratio was significantly greater in diabetic p27 +/+ mice than that in control p27 +/+ mice. In contrast, there was no difference in kidney weight to body weight ratio between diabetic and control p27 −/− mice. These results were different from those obtained in rats with short-term diabetes.

Glomerular Volume

Glomerular volume was used to assess glomerular hypertrophy. The glomerular volume of control p27 −/− mice was significantly greater than that of normal p27 +/+ mice either at 3 wk (0.50 ± 0.03 versus 0.39 ± 0.02 10⁶ μm³) or at 12 wk (0.56 ± 0.05 versus 0.35 ± 0.03 10⁶ μm³) (Figure 1). There were a 1.3-fold increase at 3 wk (0.53 ± 0.02 10⁶ μm³) and a 1.5-fold increase at 12 wk (0.53 ± 0.02 10⁶ μm³) in glomerular volume in diabetic p27 +/+ mice compared with control p27 +/+ mice (both P < 0.05). In contrast, there was no significant change in glomerular volume in diabetic p27 −/− mice either at 3 wk (0.53 ± 0.10 10⁶ μm³) or at 12 wk (0.55 ± 0.04 10⁶ μm³) compared with control p27 −/− mice.

PCNA Staining

Glomerular cell proliferation has been reported to occur in the early phase of diabetes (17). We examined whether there was a difference in cell proliferation between p27 +/+ and p27 −/− mice. PCNA staining was barely detectable in the glomeruli of both control p27 +/+ and p27 −/− mice either at 3 wk or 12 wk. Glomerular PCNA-positive cells increased in both p27 +/+ mice (5.9 ± 0.4/glomerulus) and p27 −/− mice (6.1 ± 0.5/glomerulus, NS versus p27 +/+ mice) with short-term diabetes. At 12 wk, PCNA-positive cells decreased similarly in diabetic p27 +/+ mice (1.8 ± 0.2/glomerulus) and diabetic p27 −/− mice (1.5 ± 0.2/glomerulus, NS versus p27 +/+ mice).

While there was no glomerular hypertrophy, kidney weight per body weight increased in diabetic p27 −/− mice at 3 wk (Table 1). Previous studies reported an increase in tubular cell mitosis in diabetic rats (18,19). To examine whether the kidney enlargement is due to an increase in tubulointerstitial cell proliferation, we assessed tubulointerstitial PCNA expression. There was no significant difference in the number of PCNA-positive tubulointerstitial cells

Table 1. Characteristics of study animals at 3 wk after diabetes induction

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Body Weight (g)</th>
<th>Kidney Weight (g)</th>
<th>Kidney Weight/100 g body wt</th>
<th>Plasma Glucose (mg/dl)</th>
</tr>
</thead>
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<tr>
<td>p27 +/+</td>
<td>5</td>
<td>26.7 ± 1.7</td>
<td>0.354 ± 0.038</td>
<td>1.31 ± 0.08</td>
<td>187 ± 13</td>
</tr>
<tr>
<td>CON</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>5</td>
<td>20.0 ± 1.8</td>
<td>0.348 ± 0.034</td>
<td>1.77 ± 0.17</td>
<td>560 ± 65</td>
</tr>
<tr>
<td>p27 −/−</td>
<td>5</td>
<td>32.4 ± 2.2</td>
<td>0.492 ± 0.043</td>
<td>1.51 ± 0.04</td>
<td>210 ± 17</td>
</tr>
<tr>
<td>CON</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>5</td>
<td>30.2 ± 1.5</td>
<td>0.609 ± 0.054</td>
<td>2.01 ± 0.12</td>
<td>552 ± 61</td>
</tr>
</tbody>
</table>

*CON, control; DM, diabetes.

b P < 0.05 versus CON.

a P < 0.05 versus p27 +/+.
between control p27 +/+ mice and control p27 −/− mice (3.3 ± 0.5 and 3.0 ± 0.5/high power field, respectively). In diabetic p27 +/+ and p27 −/− mice 3 wk after diabetes induction, tubulointerstitial PCNA staining was increased similarly (29.7 ± 3.0 and 30.9 ± 2.1/high power field, respectively; both \( P < 0.05 \) versus their respective controls). At 12 wk, PCNA-positive cells were significantly less (4.4 ± 0.5 and 4.1 ± 0.4 per high power field in diabetic p27 +/+ and p27 −/− mice, respectively).

**Mesangial Expansion**

We next examined whether the absence of p27 affected mesangial expansion, one of the major characteristics of diabetic nephropathy. Diabetic p27 +/+ mice showed an increase in PAS-positive mesangial matrix area by 340% at 12 wk compared with control p27 +/+ mice (Figures 2 and 3). The relative mesangial area calculated by mesangial area/glomerular area ratio also increased by 270%. In p27 −/− mice, diabetic mesangial expansion was significantly milder than that in p27 +/+ mice. Thus, mesangial area and mesangial area/glomerular area increased by 70% and 60%, respectively (both \( P < 0.05 \) versus diabetic p27 +/+ mice).

**Urine Albumin Excretion**

Urine albumin excretion, one of functional parameters in diabetic nephropathy, was significantly increased in diabetic p27 +/+ mice at 12 wk compared with control p27 +/+ mice (Figure 4). In contrast, urine albumin excretion in diabetic p27 −/− mice was not significantly different from that in control p27 −/− mice.

**Glomerular TGF-\( \beta \) Protein**

TGF-\( \beta \) is thought to mediate hypertrophy and extracellular matrix accumulation in diabetic nephropathy. We tested whether the absence of glomerular hypertrophy and amelioration of mesangial expansion in p27 −/− mice were due to reduction in TGF-\( \beta \) expression. TGF-\( \beta \) expression was assessed by immunoblot analysis of glomerular lysate (Figure 5). TGF-\( \beta \) expression increased similarly in diabetic p27 +/+ and p27 −/− mice compared with their controls.

**Glomerular Fibronectin Protein**

We next examined glomerular protein expression of fibronectin, an extracellular matrix protein and a target of TGF-\( \beta \). Diabetic p27 +/+ mice showed an increase in glomerular fibronectin protein by 67% compared with control p27 +/+ mice (Figure 6). Glomerular fibronectin protein content in control p27 −/− mice was similar to that in control p27 +/+ mice. In contrast to p27 +/+ mice, however, glomerular fibronectin did not increase in diabetic p27 −/− mice.

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**Table 2. Characteristics of study animals at 12 wk after diabetes induction**

<table>
<thead>
<tr>
<th>n</th>
<th>Body Weight (g)</th>
<th>Kidney Weight (g)</th>
<th>Kidney Weight/100 g body wt</th>
<th>Plasma Glucose (mg/dl)</th>
<th>BP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p27 +/+ CON</td>
<td>6</td>
<td>22.9 ± 2.2</td>
<td>0.276 ± 0.034</td>
<td>1.20 ± 0.05</td>
<td>166 ± 12</td>
</tr>
<tr>
<td>DM</td>
<td>6</td>
<td>19.8 ± 1.4</td>
<td>0.334 ± 0.022</td>
<td>1.71 ± 0.14( ^b )</td>
<td>357 ± 27( ^b )</td>
</tr>
<tr>
<td>p27 −/− CON</td>
<td>6</td>
<td>26.8 ± 1.6</td>
<td>0.352 ± 0.052</td>
<td>1.45 ± 0.11</td>
<td>180 ± 19</td>
</tr>
<tr>
<td>DM</td>
<td>6</td>
<td>26.2 ± 1.7( ^c )</td>
<td>0.397 ± 0.030</td>
<td>1.53 ± 0.12</td>
<td>361 ± 17( ^b )</td>
</tr>
</tbody>
</table>

\( ^a \) CON, control; DM, diabetes.

\( ^b \) \( p < 0.05 \) versus CON.

\( ^c \) \( p < 0.05 \) versus p27 +/+ DM.

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**Figure 1.** Glomerular volume increased in diabetic p27 +/+ mice but not in diabetic p27 −/− mice. (A) 3 wk after diabetes induction (\( n = 5 \)); (B) 12 wk after diabetes induction (\( n = 6 \)). CON, control; DM, diabetes. Values are mean ± SEM; \(* P < 0.05 \) versus control CON.
Effect of High Glucose on Protein Synthesis, [3H]-Leucine, and [3H]-thymidine Incorporation in Mesangial Cells from p27/H11001/H11001 and p27/H11002/H11002 Mice

To investigate the mechanism of the absence of glomerular hypertrophy in p27 -/- mice, we cultured mesangial cells from p27 +/- and p27 -/- mice under high glucose conditions. Treatment with high glucose for 5 d caused significant increases in protein content and [3H]-leucine incorporation corrected for cell number in p27 +/- mesangial cells compared with cells cultured under normal glucose (Figure 7, A and B). In contrast, these parameters significantly decreased in p27 -/- cells under high glucose conditions. Cell number decreased by 40% in p27 +/- cells and increased by 30 to 50% in p27 -/- cells, respectively, by high glucose. These changes were accompanied by a decrease in [3H]-thymidine incorporation per well in p27 +/- cells and p27 -/- cells, respectively (Figure 7C). Thus, high glucose induced cell cycle arrest and hypertrophy in p27 +/- cells, but not in p27 -/- cells.

4E-BP1 Phosphorylation in Mesangial Cells from p27 +/- and p27 -/- Mice

A previous study by Wolf et al. (12) showed that TGF-β-induced fibronectin mRNA induction was similar in p27 +/- and p27 -/- mesangial cells. In the present study, however, glomerular fibronectin protein expression was not increased in diabetic p27 -/- mice. These findings suggest that translation

**Figure 2.** Mesangial expansion is greater in diabetic p27 +/- mice than in diabetic p27 -/- mice. CON, control; DM, diabetes. Values are mean ± SEM, n = 6, *P < 0.05 versus CON; **P < 0.05 versus diabetic p27 +/-.

**Figure 3.** PAS staining at 12 wk. The glomerular tuft area and mesangial area are increased in diabetic p27 +/- mice (B) compared with control p27 +/- mice (A). The glomerular tuft did not change in diabetic p27 -/- mice (D) compared with control p27 -/- mice (C). Mesangial expansion was milder in diabetic p27 -/- mice (D) than in diabetic p27 +/- mice (B).

**Figure 4.** Urine albumin excretion increased in diabetic p27 +/- mice but not in diabetic p27 -/- mice. A 24-h urine sample for each mouse was collected in metabolic cages 12 wk after the induction of diabetes. CON, control; DM, diabetes. Values are mean ± SEM; n = 6; *P < 0.05 versus CON.
process may be impaired in p27−/− mice. Thus, we examined the phosphorylation of 4E-BP1, the inhibitory binding protein of eukaryotic initiation factor 4E (eIF4E) in mesangial cells cultured from p27+/+ and p27−/− mice. Upon phosphorylation of 4E-BP1, eIF4E is released from a heterodimeric complex and protein synthesis initiated. As shown in Figure 8, 4E-BP1 phosphorylation in p27+/+ cells cultured under high glucose for 5 d was increased compared with cells cultured under normal glucose. In control p27−/− cells, phosphorylated 4E-BP1 was increased compared with control p27+/+ cells. In contrast to p27+/+ cells, however, phosphorylated 4E-BP1 decreased in p27−/− cells under high glucose conditions.

**Discussion**

The present study shows that renal hypertrophy, glomerular hypertrophy, and albuminuria did not develop and that mesangial expansion was milder in p27−/− mice with long-term diabetes despite glomerular TGF-β upregulation. These results demonstrate the in vivo importance of p27 in the progression of diabetic nephropathy.

Glomerular hypertrophy did not occur in diabetic p27−/− mice either at 3 wk or 12 wk after diabetes induction. On the other hand, kidney weight per body weight increased significantly at 3 wk but not at 12 wk. Tubular hypertrophy is considered to be quantitatively responsible for the larger part of diabetic kidney enlargement, because it comprises the bulk of the kidney (20). Two possible explanations for kidney enlargement in p27−/− mice with short-term diabetes are considered. First, tubular cells in p27−/− mice might have behaved differently from mesangial cells and underwent hypertrophy in response to hyperglycemia. It has been shown that in the unilateral ureteral obstruction model, the absence of p21 caused interstitial cell proliferation but had no effect on tubular cells (21). In STZ-induced diabetes, the absence of p21 resulted in tubular cell hyperplasia but did not affect glomerular cells (8). These findings suggest that CKI may differently affect the cell cycle depending on the cell type. We think, however, this possibility is unlikely, because there was no increase in kidney weight per body weight ratio in p27−/− mice with long-term diabetes. An alternative explanation for the kidney enlargement in p27−/− mice at 3 wk may be tubular cell hyperplasia. In support of this hypothesis, PCNA staining increased similarly in tubulointerstitial cells of p27
and p27 mice with short-term diabetes. It has been known that cell proliferation increases in the tubulointerstitium contrasting to glomerular cells in diabetic nephropathy (18,19). A previous study by Rasch et al. (18) concluded that hyperplasia and hypertrophy participated to approximately the same extent in causing kidney enlargement in STZ-induced diabetic rats. The fact that PCNA-positive cells decreased in both diabetic p27 and p27 mice at 12 wk further supports this possibility. Taken together, cell proliferation most likely is the cause for the kidney growth in p27 and p27 mice with short-term diabetes.

Figure 7. Effect of high glucose on protein content, [3H]-leucine, and [3H]-thymidine incorporation in mesangial cells from p27 (+/-) and -/- mice. Mesangial cells were grown under high glucose (30 mM) or normal glucose (5.5 mM) for 5 d. Protein content corrected for cell number (A, n = 4). Cell number decreased in p27 (+/-) cells (2.6 to 1.5 x 10^4/well) and increased in p27 (-/-) cells (1.6 to 2.4 x 10^4/well) by high glucose treatment. [3H]-leucine incorporation corrected for cell number (B, n = 6). Cell number decreased in p27 (+/-) cells (3.2 to 2.0 x 10^5/well) and increased in p27 (-/-) cells (3.4 to 4.4 x 10^5/well) by high glucose. [3H]-thymidine incorporation (C, n = 4) was expressed per well. *P < 0.05 versus NG.

Figure 8. 4E-BP1 phosphorylation increased in mesangial cells from p27 (+/+) mice but not in cells from p27 (-/-) mice. Mesangial cells were grown under high glucose (30 mM) or normal glucose (5.5 mM) for 5 d. Cells were lysed, and the lysates were subjected to immunoblot analysis. β and γ forms of 4E-BP1 were detected with antibody which only recognizes phosphorylated form of protein (upper column). Anti-4E-BP1 antibody detects α, β, and γ forms. Quantitative analysis of phosphorylated γ isoform is shown below. Values are mean ± SEM; n = 3; *P < 0.05 versus CON; **P < 0.05 versus control p27 (+/+).

+/- and p27 -/- mice with short-term diabetes. It has been known that cell proliferation increases in the tubulointerstitium contrasting to glomerular cells in diabetic nephrpathy (18,19). A previous study by Rasch et al. (18) concluded that hyperplasia and hypertrophy participated to approximately the same extent in causing kidney enlargement in STZ-induced diabetic rats. The fact that PCNA-positive cells decreased in both diabetic p27 (+/) and p27 (-/-) mice at 12 wk further supports this possibility. Taken together, cell proliferation most likely is the cause for the kidney growth in p27 (+/) and p27 (-/-) mice with short-term diabetes.

The mechanism for the absence of glomerular hypertrophy in p27 -/- mice was assessed using cultured mesangial cells. Mesangial cells are the key component for glomerular hypertrophy and extracellular matrix accumulation leading
to glomerulosclerosis and the decline of glomerular filtration. A previous study by Wolf et al. (11) suggested the importance of p27 in hypertrophy of mesangial cells caused by high glucose. They demonstrated using antisense oligonucleotide that p27 was required for mesangial cell hypertrophy. In the present study, protein synthesis and leucine incorporation corrected for cell number increased under high glucose conditions in p27 +/- mesangial cells, whereas both of these parameters decreased in p27 +/- cells. Thymidine incorporation per well decreased and increased in p27 +/- and p27 +/- cells, respectively, after 5-d treatment with high glucose. Thus, protein:DNA ratio, a measure of cellular hypertrophy, increased in p27 +/- and decreased in p27 +/- cells. These results are in accord with a recent study by Wolf et al. (12) using mesangial cells from p27 +/- mice. It has been reported that high glucose stimulates cell proliferation at 24 to 48 h and causes an antiproliferative effect thereafter (22). The glucose-induced antiproliferation has been shown to be mediated by TGF-β. Thus, anti-TGF-β neutralizing antibody stimulated the cell cycle progression toward S phase and reversed mesangial cell hypertrophy (22,23). The mechanisms of TGF-β to inhibit cell cycle progression have been shown to be down-regulation of cyclin D1, cyclin E, cyclin A, CDK4, or CDK2 depending on the cell type (24–27). TGF-β also prevents down-regulation of p27 and induces p21 and p15Ink4B in a variety of cell types (26,28). A neutralizing anti-TGF-β antibody partially prevented upregulation of p27 in mesangial cells under high glucose (11).

Wolf et al. (12) reported that TGF-β expression increased similarly in p27 +/- and p27 +/- mesangial cells under high glucose conditions. In the present study also, glomerular TGF-β expression increased similarly in p27 +/- and p27 +/- mice. Monkawa et al. (29) recently reported that TGF-β-induced cellular hypertrophy is attenuated in p27 +/- cells. In the study by Wolf et al., reconstituting p27 by transient or stable transfection in p27 +/- cells increased protein synthesis and restored G1 phase arrest. Collectively, p27 appears to play an important role downstream of TGF-β in high glucose-induced cell cycle arrest and hypertrophy both in vitro and in vivo.

We previously reported that extracellular signal-regulated protein kinase (ERK) activity is increased in diabetic glomeruli (30). It has recently been demonstrated that Ras/Raf/ERK pathway stimulates cell cycle progression by inducing cyclin D1 and cyclin A and by downregulating p27 (31–33). As stated above, high glucose initially stimulates proliferation of cultured mesangial cells (22). It is also known that there is transient low-grade mesangial cell proliferation in diabetic nephropathy (34). The early phase of mesangial proliferation under high glucose seen both in vivo and in vitro is likely due to the activation of ERK. In the subsequent induction of hypertrophy as well, ERK is thought to play a role. Both entry into G1 phase and arrest at late G1 phase are necessary for cells to undergo hypertrophy (3). ERK probably mediates the former, and the latter is mediated by TGF-β. A recent study suggests that ERK is also responsible for TGF-β upregulation in mesangial cells (35).

Increased extracellular matrix is another characteristic feature of diabetic nephropathy, and it contributes to diabetic glomerular hypertrophy and subsequent glomerulosclerosis. Synthesis of matrix molecules such as fibronectin, type IV collagen, and laminin has been demonstrated to be increased (36). TGF-β is the mediator of extracellular matrix accumulation as well as hypertrophy in diabetic kidney. In the present study, TGF-β protein expression increased in the glomeruli of both diabetic p27 +/- and p27 +/- mice. Thus, milder glomerular expansion in p27 +/- mice is not due to the lack of the increase in TGF-β expression but is considered to be due to the absence of p27. On the other hand, glomerular fibronectin protein expression increased in p27 +/- mice but not in p27 +/- mice. Of note, Wolf et al. showed that fibronectin mRNA was induced similarly by TGF-β in p27 +/- and p27 +/- mesangial cells cultured under high glucose. Together with the present results, translation of fibronectin is considered to be impaired in p27 +/- mice. To investigate the mechanism of the impaired protein translation, we examined 4E-BP1, the translational inhibitor, in mesangial cells cultured from p27 +/- and p27 +/- mice. Phosphorylation of 4E-BP1 causes the release of eIF4E from the eIF4F complex, leading to the upregulation of the translation of a subset of mRNAs (37). Thus phosphorylation of 4E-BP1 is critical in the regulation of the initiation of protein synthesis. Phosphorylation of 4E-BP1 increased in p27 +/- cells cultured under high glucose conditions for 5 d. In control p27 +/- cells, the level of phosphorylated 4E-BP1 was higher than that in control p27 +/- cells and decreased under high glucose conditions. Thus impaired fibronectin translation in p27 +/- mice may be due to the inhibition of 4E-BP1 phosphorylation. It remains to be determined why 4E-BP1 phosphorylation under diabetic state is impaired in p27 +/- mice.

Albuminuria, a functional parameter of diabetic nephropathy, increased in diabetic p27 +/- mice but not in p27 +/- mice. The absence of albuminuria may be related to the milder glomerular expansion in p27 +/- mice. Long-term studies are needed whether the absence of p27 ameliorates the decline of GFR and glomerulosclerosis. In this regard, BP tended to be lower in diabetic p27 +/- mice compared with control p27 +/- mice or diabetic p27 +/- mice although statistical significance was not reached. BP is an important factor affecting the severity of renal injury caused by various stimuli including diabetes. The number of mice examined in the present study was small; therefore, the possibility cannot be ruled out that the attenuation of diabetic kidney injury in p27 +/- mice was partly due to the lower BP.

In conclusion, the present study demonstrates that p27 is required for the progression of diabetic nephropathy. p27 has been shown to safeguard against excessive cell proliferation in experimental glomerulonephritis and ureteral obstruction (38). While p27 has beneficial effects in these specific pathophysiological settings, it appears to play a detrimental role in diabetic nephropathy. Although TGF-β is considered to be a key me-
diator of diabetic nephropathy and various strategies have been proposed to inhibit the TGF-β loop, we showed that the effect of TGF-β could be blocked at the level of cell cycle regulatory protein. TGF-β has antiinflammatory and antimitogenic actions, and nonselective blockade may be deleterious; therefore, p27 may be a better target for the prevention and/or treatment of diabetic nephropathy.

Acknowledgments

We thank Nippon Roche Research Center for providing mice heterozygous for the p27. Financial support has been provided by Ministry of Education, Science, and Culture, Japan (10670757, 12770610, 12770401, 13770404, 14570772) and Pharmacia-Upjohn Fund for Growth and Development Research.

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