Galectin-9 Inhibits Glomerular Hypertrophy in db/db Diabetic Mice via Cell-Cycle–Dependent Mechanisms

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Galectins are β-galactoside–binding lectins that are involved in various biologic processes, such as apoptosis, cell proliferation, and cell-cycle regulation. Galectin-9 (Gal-9) was identified previously and demonstrated to have apoptotic potential to thymocytes in mice and activated CD8⁺ T cells in nephrototoxic serum nephritis model. In this study, the effect of Gal-9 on G1-phase cell-cycle arrest, one of the hallmark pathologic changes in early diabetic nephropathy, was investigated. Eight-week-old male db/db mice received injections of recombinant Gal-9 or vehicle for 8 wk. The injection of Gal-9 into db/db mice significantly inhibited glomerular hypertrophy and mesangial matrix expansion and reduced urinary albumin excretion. Gal-9 reduced glomerular expression of TGF-β1 and the number of p27kip1- and p21cip1-positive cells in glomeruli. Double staining with nephrin and type IV collagen revealed that podocytes were mainly positive for p27kip1. For further confirming the cell-cycle regulation by Gal-9, conditionally immortalized mouse podocyte cells were cultured under 5.5 and 25 mM d-glucose supplemented with Gal-9. Cell-cycle distribution analyses revealed that Gal-9 maintained further progression of cell cycle from the G1 phase. Gal-9 reversed the high-glucose–mediated upregulation of p27kip1 and p21cip1 and inhibited cell-cycle-dependent hypertrophy, i.e., reduced [³H]proline incorporation. The data suggest that Gal-9 plays a central role in inducing their successful progression from G1 to G2 phase by suppressing glomerular expression of TGF-β1 and inhibition of cyclin-dependent kinase inhibitors. Gal-9 may give an impetus to develop new therapeutic tools targeted toward diabetic nephropathy.


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apoptotic effect in various cell types include Gal-7 in keratinocytes (28,29), Gal-8 in cancer cells (30), and Gal-12 in adipocytes and neoplastic cells (31–33). However, Gal-3 has an antiapoptotic effect in T cells and breast cancer cells (34,35). In terms of cell-cycle regulation, Gal-1 has no significant effect in normal cells, and it acts as a negative cell-cycle regulator in T cells (15,36) and induces proliferation of endothelial cells and fibroblasts (37,38). In contrast, Gal-1 causes S/G2 cell-cycle arrest and apoptosis in various cancer cells, such as of breast and neural tissues (15).

In view of the above biologic effects of various galectins, it is conceivable that Gal-9 may act as an essential cell-cycle regulator. At present, there are numerous ongoing studies investigating the effect of galectins on cell cycle in various cancer cells. The cell cycle is dysregulated in the diabetic state, and G1-phase arrest is believed to be responsible for the high-glucose-induced cellular hypertrophy and increase in the de novo protein synthesis and consequent accumulation of extracellular matrix proteins, as typically seen in diabetic nephropathy (39,40). There is a growing body of evidence that specific cyclin-dependent kinase (CDK) inhibitors, p27Kip1 and p21Cip1, are critically involved in hypertrophy of mesangial cells that are exposed to high glucose ambience (41,42) and in experimental type 1 and 2 diabetes (43,44) and also in p27Kip1 knockout (45). The inhibition of TGF-β-mediated hypertrophy in cultured mesangial cells derived from p27Kip1 and p21Cip1 double null (−/−) mice also support the notion that CDK inhibitors are critically involved in hypertrophy of mesangial cells (46). Our study describes the ameliorative effect of the Gal-9 on the progression of diabetic nephropathy in db/db mice, which seems to be related to its modulation of the cell cycle via CDK inhibitors: p27Kip1 and p21Cip1.

Materials and Methods

Animals and Experimental Protocol

Eight-week-old male db/db mice that lack the hypothalamic leptin receptor (47), a model of type 2 diabetes, were purchased (Clea Co. Ltd., Tokyo, Japan) and used for various studies. These mice manifest hyperglycemia, hyperinsulinemia, hyperleptinemia, hyperlipidemia, and obesity commencing at 4 to 6 wk of age and albuminuria at 6 wk after birth (48,49). The db/db mice were divided into two groups: Diabetic db/db mice that were treated with PBS that contained 1 mM dithiothreitol (DTT; PBS–DTT buffer; db/db, n = 10) and db/db mice that were treated with recombinant Gal-9 in PBS–DTT buffer (db/db + Gal-9, n = 10). Nondiabetic db/m mice were also divided into two groups: db/m mice that were treated with PBS–DTT buffer (db/m, n = 10) or treated with recombinant Gal-9 in PBS–DTT buffer (db/m + Gal-9, n = 10). Gal-9 (1 mg/kg) or PBS–DTT buffer was administered intraperitoneally three times a week for a period of 8 wk, and the animals were killed at 16 wk of age. Blood and tissue samples were harvested and processed for various studies. Blood samples were collected from tail vein after a 12-h fast. The mice were placed in individual metabolic cages for 24-h urine collection. BP was measured by tail-cuff method (BP-98A; Softron Corp., Tokyo, Japan).

Biochemical Analyses

Blood glucose, hemoglobin A1c, serum creatinine, plasma insulin, and daily urinary albumin excretion were measured at 8, 12, and 16 wk of age. Total cholesterol, triglyceride, and free fatty acid levels were also measured at 16 wk of age. Urinary albumin concentration was measured by nephelometry (Organon Teknika-Cappel, Durham, NC) and insulin levels were measured by RIA (Linco Research Inc., St. Charles, MO).

Preparation of Recombinant Mouse Gal-9

Production of recombinant mouse Gal-9 was carried out using pTrcHis2 vector (Invitrogen, San Diego, CA) as described previously (9,21,23). After pTrcHis2/G9 was transformed into the TOP10 bacterial host (Invitrogen), a single colony was picked and grown in Luria-Bertani’s medium. Protein synthesis was induced with the addition of 1 mmol/L isopropyl-β-D-thiogalacto-pyranoside (Sigma, St. Louis, MO). The cell pellets were lysed in PBS that contained 1 mM DTT, 1.25% Triton X-100, 10 mM benzamidine, 10 mM e-amino-n-caproic acid, and 2 mM PMSF. Supernatants were applied to a 15-mL lactosyl-Sepharose column (Sigma) and eluted with PBS that contained 1 mM DTT and 200 mmol/L lactose. The eluted fractions were collected and dialyzed against PBS buffer that contained 1 mM DTT. The endotoxin concentration in the purified Gal-9 was <10 EU/mg proteins, as assessed by Limulus Amebocyte Lysate Assay (Seikagaku Corp., Tokyo, Japan).

Morphologic Studies

Renal tissues were fixed in 10% paraformaldehyde and embedded in paraffin, and 4-μm-thick sections were prepared. The sections were stained with periodic acid-Schiff (PAS). Glomerular tuft and mesangial matrix area were measured with image analysis software (Optimas version 6.5; Media Cybernetics, Silver Spring, MD). The cross-section that yielded the maximum diameter of the glomerulus was photographed and converted into a digital image, and ultrastructural examination of the glomeruli was carried out using tissues that were processed for electron microscopy (50).

Immunohistochemistry

Four-micrometer-thick sections of formalin-fixed, paraffin-embedded tissues were deparaffinized and rehydrated, and endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide and methanol. Sections were pretreated by microwave for 20 min in citrate buffer for antigen retrieval. Non-specific binding was blocked by incubation for 30 min in 10% rabbit serum. The tissues then were incubated with anti-p27Kip1, p21Cip1, TGF-β1, and vascular endothelial growth factor (VEGF) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). After PBS wash, sections were incubated with a biotinylated secondary antibody and ABC-Elite Reagent (Vector Laboratories, Burlingame, CA). The percentage of p27Kip1- and p21Cip1-positive cells in all glomerular cells was evaluated, and 50 glomeruli in each animal were analyzed. For evaluating the expression of TGF-β1, 50 glomeruli were examined under high magnification (×400), and the TGF-β1–positive area in a glomerulus was measured using Optimas version 6.5. The TGF-β1–positive area of each glomerulus was divided by the mean value of TGF-β1–positive areas in db/db mice and indicated as relative area.

Four-micrometer frozen sections were prepared and fixed with cold acetone for 3 min. For evaluation of mesangial matrix accumulation and identification of p27Kip1- or p21Cip1–positive glomerular cells, sections were treated with 1:100 diluted rabbit anti–type IV collagen, p27Kip1 and p21Cip1 polyclonal antibody (Santa Cruz Biotechnology), and guinea pig anti-nephrin polyclonal antibody (Progen, Heidelberg, Germany) followed by incubation with goat anti-rabbit and anti-mouse IgG conjugated with FITC or rhodamine (Chemicon, Temecula, CA).
Digital images were obtained using confocal laser fluorescence microscope (LSM-510; Carl Zeiss, Jena, Germany), and type IV collagen expression was quantified with the formula (density indicated by 0 to 255 in gray scale × positive area μm²) as described previously (51).

**Northern Blotting and Quantitative Real-time PCR**

Total RNA was isolated from kidney tissue of mice and cells using QIAzol Reagent (Qiagen, Hilden, Germany). Twenty micrograms of total RNA was subjected to 2.2 M formaldehyde 1% agarose gel electrophoresis, and capillary was transferred to the Hybond XL nylon membranes (Amersham Biosciences, Piscataway, NJ). The membranes were hybridized with [α-32P]dCTP-radiolabeled mouse α1(IV) collagen, α5(IV) collagen, TGF-β1, VEGF, and human β-actin (1× 10⁶ cpm/ml) cDNA (American Type Culture Collection [ATCC], Rockville, MD) at 68°C in ExpressHyb Hybridization Solution (Clontech, Palo Alto, CA) for 1 h. Filters were washed at high stringency conditions (four times in 1× SSC/0.1% SDS at 20°C, followed by two times at 50°C in 0.1× SSC/0.1% SDS). Autoradiogram was prepared using Bio-Imaging Analyzer System (BAS-1800II; Fujifilm, Tokyo, Japan).

For quantitative real-time PCR analysis, cDNA were synthesized from 1 μg of total RNA and analyzed using LightCycler-FastStart DNA master SYBR Green I system (Roche Diagnostic Co., Basel, Switzerland) and specific primers. The relative abundance of mRNA was standardized with β-actin mRNA as the invariant control. The primers used were as follows: Mouse TGF-β1 5'-AACACGCACTCATTGAG-3' and 5'-TATTCCTGCTCCTGTT-3' (Nihon Gene Research Lab's Inc., Sendai, Japan).

**Cell Culture**

For propagating mouse podocyte cell (MPC) lines (gift from Prof. Peter Mundel, Albert Einstein College of Medicine, Bronx, NY) (52), the cells were cultured on type I collagen–coated flasks (BD Falcon, San Jose, CA) with RPMI 1640 (Invitrogen, Carlsbad, CA) that contained 10% FCS (Invitrogen), 10% nonfat dry milk and 50 U/ml recombinant mouse IFN-γ (BD Biosciences, Palo Alto, CA) at 33°C. After 90% confluence, the cells were subjected to the following studies: Cell cycle analysis, [3H]thymidine incorporation and Western blot analysis.

**Measurement of Cell Number and Cellular Protein**

Cells were washed with PBS and trypsinized for counting the cell number in a Coulter counter (model Z1; Beckman Coulter, Fullerton, CA) with 100-μm aperture (Beckman Coulter). Cells were also lysed, and the total protein content was determined by Lowry method (Biorad, Hercules, CA).

**Western Blotting**

At the end of MPC and MES culture, the cells were washed with PBS and lysed with a buffer (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, 10 mM benzamidine-HCl, 10 mM 3-mercaptoethanol, 2 mM PMSF, and 1% Triton X-100). Forty micrograms of protein was subjected to SDS-PAGE under reducing conditions and electrophoresed onto Hybond P polyvinylidene difluoride membranes (Amersham Biosciences). The membrane blots were immersed in a blocking solution that contained 5% nonfat dry milk and TBS-T (0.05% Tween 20, 20 mM Tris-HCl, and 150 mM NaCl [pH 7.6]). Then, membranes were incubated individually with rabbit polyclonal anti-p27Kip1 (1:100 dilution), anti-p21Cip1 (1:100 dilution; Santa Cruz Biotechnology), and anti-actin antibody (1:500 dilution; Sigma). Then they were incubated with anti-rabbit IgG conjugated with horseradish peroxidase (1:20,000 dilution; Amersham) for polyclonal antibodies and anti-mouse IgG conjugated with horseradish peroxidase (1:20,000 dilution; Amersham) for monoclonal antibodies. The blots were then washed three times with TBS-T, immersed in ECL Plus Western Blotting Detection Reagents (Amersham), and then exposed to Hyperfilm ECL (Amersham).

**Cell-Cycle Analysis by Laser Scanning Cytometry**

MPC seeded on type I collagen–coated chamber slides (BD Falcon) were fixed with 100% ethanol for 15 min. The cells were treated with 200 μg/ml DNAse free-RNAse A (Sigma) for 15 min at 37°C, and then nuclear DNA was stained with 50 μg/ml propidium iodide (Sigma) in the dark. Stained cells were analyzed with a laser scanning cytometer (Olympus Optical Co. Ltd., Tokyo, Japan) by measuring total and peak intensity of propidium iodide fluorescence in each nucleus (53). We analyzed approximately 3000 unperturbed MPC populations and generated a detailed profile of the cell cycle, i.e., percentage of the cells in G0/G1, S, G2, and M. Cell-cycle analysis was performed independently three times.

**Statistical Analyses**

Data were expressed as the mean ± SEM and analyzed by unpaired t test or a one-way ANOVA by Fisher t test when multiple comparisons against the control were required. We used χ² test with Yates correction for 2×2 tables to compare the categorical data. P < 0.05 was regarded as statistically significant. The data were analyzed with Dr. SPSS II for Windows release 11.0.1J.

**Results**

**Administration of Gal-9 Reduces Urinary Albumin Excretion in db/db Mice**

The baseline (8 wk of age) and final (16 wk of age) body weights of db/db mice were significantly greater than those of db/m mice (Table 1). The increase in body weight over the experimental period was similar in db/db and the db/db+Gal-9 group. There were no significant differences in BP among four groups throughout the study period. The diabetic db/db mice remained hyperglycemic, and hemoglobin A1c levels were significantly higher in db/db mice compared with db/m littermates. At the end of the study, db/db mice had significant
urinary albumin excretion in
at 12 and 16 wk of age. Gal-9 treatment significantly reduced

/db/db Mice
Gal-9 Infusion Ameliorates Glomerular Hypertrophy in
/db/db mice. However, the average kidney-to-body weight ratios were higher in/db/m mice, and there were no statistically significant differences between/db/db mice and/db/db+Gal-9 mice, because diabetic/db/db mice were much heavier than/db/m littermates. Urinary albumin excretion was significantly increased in/db/db mice compared with/db/m mice at 12 and 16 wk of age. Gal-9 treatment significantly reduced urinary albumin excretion in/db/db mice at 12 and 16 wk of age (Figure 1).

Gal-9 Infusion Ameliorates Glomerular Hypertrophy in/db/db Mice

At the end of the study, PAS-positive mesangial matrix area and glomerular size of/db/db mice were significantly increased compared with/db/m mice (Figure 2, A through D). Morphometric analysis clearly indicated that Gal-9 significantly reduced both glomerular cross-sectional area and the mesangial matrix index, i.e., the ratio of mesangial matrix area divided by the tuft area (Figure 2, E and F). The infusion of Gal-9 into/db/m mice revealed no effects on the electron microscopic findings (Figure 3, A and B). Amelioration in the mesangial expansion by Gal-9 treatment was confirmed by electron microscopic examination (Figure 3, D and F). In addition, the cellular hypertrophy, especially in podocytes, was noted in/db/db mice (Figure 3C), whereas such hypertrophic changes in glomeruli

Table 1. Various parameters of/db/m and db/db mice after Gal-9 treatmenta

<table>
<thead>
<tr>
<th></th>
<th>db/m (n = 10)</th>
<th>db/m+Gal-9 (n = 10)</th>
<th>Db/db (n = 10)</th>
<th>db/db+Gal-9 (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight at 8 wk (g)</td>
<td>27.0 ± 0.40</td>
<td>28.3 ± 0.52</td>
<td>42.3 ± 0.64b</td>
<td>42.2 ± 0.71b</td>
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<tr>
<td>Body weight at 16 wk (g)</td>
<td>29.9 ± 0.40</td>
<td>29.6 ± 0.63</td>
<td>44.6 ± 1.73b</td>
<td>43.6 ± 1.17b</td>
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<td>Systolic BP at 8 wk (mmHg)</td>
<td>131 ± 3.42</td>
<td>128 ± 1.79</td>
<td>126 ± 2.91</td>
<td>129 ± 1.36</td>
</tr>
<tr>
<td>Diastolic BP at 8 wk (mmHg)</td>
<td>85.2 ± 2.46</td>
<td>79.1 ± 3.57</td>
<td>76.2 ± 4.39</td>
<td>79.5 ± 4.32</td>
</tr>
<tr>
<td>Systolic BP at 16 wk (mmHg)</td>
<td>126 ± 5.14</td>
<td>128 ± 4.46</td>
<td>138 ± 3.37</td>
<td>128 ± 2.18</td>
</tr>
<tr>
<td>Diastolic BP at 16 wk (mmHg)</td>
<td>83.2 ± 3.51</td>
<td>81.9 ± 2.36</td>
<td>91.5 ± 4.35</td>
<td>82.2 ± 2.22</td>
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<tr>
<td>Hemoglobin A1c (%)</td>
<td>2.98 ± 0.03</td>
<td>3.12 ± 0.19</td>
<td>9.61 ± 0.23b</td>
<td>9.67 ± 0.02b</td>
</tr>
<tr>
<td>Fasting plasma glucose at 8 wk (mmol/L)</td>
<td>2.70 ± 0.22</td>
<td>2.53 ± 0.98</td>
<td>16.5 ± 1.54b</td>
<td>19.0 ± 1.20b</td>
</tr>
<tr>
<td>Fasting plasma glucose at 16 wk (mmol/L)</td>
<td>5.80 ± 0.50</td>
<td>6.16 ± 0.72</td>
<td>32.6 ± 1.40b</td>
<td>31.2 ± 0.90b</td>
</tr>
<tr>
<td>Insulin at 16 wk (pmol/L)</td>
<td>2.0 ± 0.1</td>
<td>2.2 ± 0.3</td>
<td>9.1 ± 1.0b</td>
<td>8.2 ± 1.0b</td>
</tr>
<tr>
<td>Serum creatinine (µmol/L)</td>
<td>7.0 ± 1.2</td>
<td>6.9 ± 1.1</td>
<td>8.1 ± 1.3</td>
<td>9.0 ± 1.6</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>1.84 ± 0.11</td>
<td>1.97 ± 0.16</td>
<td>3.07 ± 0.27c</td>
<td>2.83 ± 0.20c</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>0.19 ± 0.04</td>
<td>0.21 ± 0.03</td>
<td>0.67 ± 0.15</td>
<td>0.66 ± 0.14</td>
</tr>
<tr>
<td>Free fatty acid (µEq/L)</td>
<td>774 ± 50.3</td>
<td>696 ± 41.4</td>
<td>1278 ± 89.7c</td>
<td>1239 ± 45.6c</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>0.19 ± 0.007</td>
<td>0.18 ± 0.004</td>
<td>0.24 ± 0.005c</td>
<td>0.22 ± 0.004cd</td>
</tr>
<tr>
<td>Kidney/body weight (%)</td>
<td>0.68 ± 0.02</td>
<td>0.63 ± 0.01</td>
<td>0.52 ± 0.01</td>
<td>0.52 ± 0.01</td>
</tr>
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</table>

aNondiabetic/db/m (db/m) and diabetic/db/db mice (db/db) were treated with PBS-dithiothreitol buffer, and db/m+Gal-9 and db/db+Gal-9 were treated with recombinant Gal-9. Parameters are recorded at baseline (8 wk of age) and end of the study (16 wk of age). Gal-9, galectin-9.

bP < 0.01 versus db/m mice.

P < 0.05 versus db/m mice.

dP < 0.05 versus db/db mice.

hyperinsulinemia and increased levels of cholesterol and free fatty acid compared with/db/m mice. The administration of Gal-9 did not alter the glucose levels and lipid profile in/db/db mice. The final kidney weight was significantly higher in/db/db compared with/db/m mice, and renal hypertrophy was ameliorated in Gal-9–treated/db/db mice. However, the average kidney-to-body weight ratios were higher in/db/m mice, and there were no statistically significant differences between/db/db mice and/db/db+Gal-9 mice, because diabetic/db/db mice were much heavier than/db/m littermates. Urinary albumin excretion was significantly increased in/db/db mice compared with/db/m mice at 12 and 16 wk of age. Gal-9 treatment significantly reduced urinary albumin excretion in/db/db mice at 12 and 16 wk of age (Figure 1).

Figure 1. Injection of galectin-9 (Gal-9) reduces albuminuria in diabetic mice. ELISA specific for mouse albumin was used to evaluate 24-h urine albumin excretion. The increased excretion of albumin in/db/db mice is reduced at 12 and 16 wk of age by repeated injection of recombinant Gal-9. Data are mean ± SEM; *P < 0.01 versus db/m mice; **P < 0.05 versus db/db mice.

were not observed in/db/db mice that were treated with Gal-9 (Figure 3E).

Gal-9 Infusion Reduced Expression of α1 (IV) and α5 (IV) Collagen and TGF-β1 but not VEGF Proteins

Northern blot analyses of kidney cortex revealed that α1 (IV) and α5 (IV) collagen mRNA levels were significantly increased, more than four-fold in/db/db mice compared with/db/m mice (Figure 4, A and B). Gal-9 treatment prevented the increase in α1 (IV) and α5 (IV) collagen mRNA expressions in/db/db mice.
Besides mRNA expression of type IV collagen chains, the immunofluorescence intensity of type IV collagen quantified by morphometric analysis also showed the accumulation of type IV collagen in glomeruli was significantly reduced with the treatment of Gal-9 (Figure 4, C through F and K). mRNA expression of growth factors that mediate the progression of diabetic nephropathy, e.g., TGF-β1 and VEGF, also significantly upregulated in db/db mice compared with db/m mice (Figure 4, A and B). However, the treatment with Gal-9 did not alter the mRNA expression of TGF-β1 and VEGF in renal cortex. We examined the expression level of TGF-β1 and VEGF proteins in renal glomeruli by using immunohistochemistry. TGF-β1 protein levels in renal glomeruli increased in diabetic db/db mice compared with control db/m mice (Figure 4, A and B). However, the treatment with Gal-9 significantly inhibited the glomerular expression of TGF-β1 protein in diabetic db/db mice (Figure 4, G through J and L). VEGF protein levels in renal glomeruli increased in diabetic db/db mice compared with control db/m mice. However, Gal-9 treatment revealed no effect on the expression of VEGF protein in diabetic db/db mice (data not shown).

**Gal-9 Decreased Glomerular p27Kip1- and p21Cip1-Positive Cells in db/db Mice**

To gain insight into the ameliorative effect of Gal-9 on diabetic nephropathy, we evaluated expression of CDK inhibitors p27Kip1 and p21Cip1 (Figure 5, A through H). Glomerular p27Kip1- and p21Cip1-positive cells were significantly increased in db/db mice compared with db/m mice (Figure 5, I and J). Although tubular cells revealed positive staining for p27Kip1 and p21Cip1, there was no significant difference between db/db and db/m mice (data not shown). The treatment with Gal-9 recombinant protein significantly reduced both p27Kip1- and p21Cip1-positive glomerular cells (Figure 5, D and H). To identify which glomerular cells are associated with positive nuclear staining of p27Kip1, we investigated the expression of p27Kip1, nephrin, and type IV collagen using double immunofluorescence studies. p27Kip1-positive glomerular cells were mainly observed in extracapillary space and adjacent with capillary lining, which was visualized by nephrin and type IV collagen immunostaining (Figure 5K). Although part of the mesangial and glomerular cells were also positive for p27Kip1, we demon-
mice that were treated with Gal-9 (B), 

To assess cellular hypertrophy/hyperplasia of MPC and MES, we assessed DNA and protein synthesis by \[^{3}H\]thymidine and \[^{3}H\]proline incorporation. In MPC culture, \[^{3}H\]thymidine incorporation and total cellular protein amount without statistical significance (Figure 6, F and H). Hyperosmotic treatment with MN did not alter the \[^{3}H\]thymidine and \[^{3}H\]proline incorporation.

**Gal-9 Reduces \[^{3}H\]proline Incorporation**

In MPC culture, high glucose significantly upregulated both \[^{3}H\]proline incorporation and total cellular protein amount without statistical significance (Figure 6, F and H). Hyperosmotic treatment with MN did not alter the \[^{3}H\]thymidine and \[^{3}H\]proline incorporation.

**Gal-9 Reduces \[^{3}H\]proline Incorporation**

In MES culture, \[^{3}H\]proline incorporation was significantly elevated compared with NG, HG, or MN, and treatment with Gal-9 also showed no effect on \[^{3}H\]proline incorporation and total cellular protein (Figure 6, B and D). In MES culture, \[^{3}H\]thymidine incorporation and cell number under high-glucose conditions were significantly reduced compared with NG, NG + Gal-9, and MN, and treatment with Gal-9 showed partial recovery of \[^{3}H\]thymidine incorporation and cell number with statistical significance (Figure 6, E and G). \[^{3}H\]proline incorporation and cellular proteins under high-glucose conditions were significantly elevated compared with NG, NG + Gal-9, and MN, and treatment with Gal-9 showed certain inhibitory effects on \[^{3}H\]proline incorporation and total cellular protein amount without statistical significance (Figure 6, F and H). Hyperosmotic treatment with MN did not alter the \[^{3}H\]thymidine and \[^{3}H\]proline incorporation.

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Figure 4. Gal-9 reduces gene expression of α1 (IV) and α5 (IV) collagens in renal cortex and protein expression of type IV collagens and TGF-β1 expression in renal glomeruli. Representative Northern blot is shown (A), and densitometric analyses are shown (B). The injections of Gal-9 significantly reduce mRNA expression of α1 (IV) and α5 (IV) collagen; however, TGF-β1, and VEGF mRNA expression is unaltered. By immunofluorescence microscopy, the intensity of type IV collagen signal significantly increases in glomeruli in db/db mice compared with db/m mice (E and C). Gal-9 treatment significantly reduces intensity of type IV collagen (E, F, and K). By immunohistochemistry, TGF-β1 protein levels in renal glomeruli increase in diabetic db/db mice compared with control db/m mice (G and I). Gal-9 treatment significantly inhibits the glomerular expression of TGF-β1 protein in diabetic db/db mice (I, J, and L). Data are mean ± SEM; *P < 0.01 versus db/m mice; **P < 0.01 versus db/db mice.
cose stimulates the expression of TGF-β1 (46,54) and connective tissue growth factor (41,55) during mesangial cell hypertrophy, a well-recognized hallmark of diabetic nephropathy (39). The upregulated expression of TGF-β1 leads to increased transcriptional activity of cyclin D and induction of CDK inhibitors through transcriptional and posttranslational mechanisms. Although mesangial cells can reenter the cell cycle, they fail to progress through G1/S phase because of the induction of CDK inhibitors. Besides mesangial cells, recent studies have demonstrated that podocyte hypertrophy is observed in differentiated podocyte cell line and Zucker fatty rats (56). In addition, increase in p21Cip1 and p27Kip1 has been described in podocytes in Zucker fatty rats, which is also seen in db/db mice in our studies (56). In type 1 and 2 diabetes, a decrease in podocyte number is well correlated with both microalbuminuria and progression of diabetic nephropathy (57). High glucose induces the expression of TGF-β1, and it in turn stimulates the expression of the CDK inhibitors p21Cip1 and p27Kip1, although high-glucose–mediated induction of p27Kip1 is to some extent independent of TGF-β1 pathway (58). Thus, one can conclude that high-glucose–mediated podocyte injury associated with G1-phase cell-cycle arrest seems to be another major player in the pathogenesis of diabetic glomerulopathy.

In db/db mice, chronic administration of Gal-9 reduced albuminuria and inhibited glomerular hypertrophy and accumulation of extracellular matrix. Injection of Gal-9 inhibited both
Figure 6. Effect of Gal-9 on high-glucose–induced hypertrophy of mouse podocytes (MPC). We cultured differentiated MPC and mouse mesangial cell line (MES). In MPC culture, DMEM that contained 0.5% FCS for 48 h and mature podocytes were incubated with 5.5 mM normal glucose (NG), NG with 1 μM Gal-9 (NG+Gal-9), 25 mM high glucose (HG), HG with 1 μM Gal-9 (HG+Gal-9), and 5.5 mM NG plus 19.5 mM mannitol (MN) for 6 d. Hypertrophy of MPC was assessed by [3H]thymidine and cell number (A and C) and by [3H]proline incorporation and cellular total protein (B and D). There are no differences in [3H]thymidine incorporation and cell number per well, whereas protein synthesis significantly increased by HG treatment, which is reduced with the addition of Gal-9, as assessed by total protein and incorporation of [3H]proline. In MES culture, [3H]thymidine incorporation and cell number under HG conditions is significantly reduced compared with NG, NG+Gal-9, and MN, and treatment with Gal-9 shows some recovery of [3H]thymidine incorporation and cell number without statistically significant difference (E and G). [3H]proline incorporation and cellular proteins under HG conditions is significantly elevated compared with NG, NG+Gal-9, and MN, and treatment with Gal-9 shows inhibitory effects on [3H]proline incorporation and cellular proteins, although it is not statistically significant (F and H). Data are mean ± SEM; *P < 0.01 versus NG; **P < 0.01 versus HG.
mRNA and protein expression of type IV collagen and glomerular expression of TGF-β1 protein. In db/db mice, immunohistochemistry clearly indicated that p27Kip1- and p21Cip1-positive cells predominantly increased in podocytes compared with nondiabetic db/m mice. Although the cell-cycle arrest and cellular hypertrophy in mesangial cells in diabetic nephropathy were well documented in the literature, we postulated that podocytes also are involved in such process. The administration of Gal-9 reduced p27Kip1- and p21Cip1-positive cells in glomeruli in db/db mice. The in vivo data suggested that Gal-9 ameliorated early diabetic nephropathy via inhibition of TGF-β1 as well as cell-cycle–dependent pathways.

To confirm further the cell-cycle–dependent therapeutic mechanism of Gal-9 under high glucose condition, we used MPC (52). Most of the standard cell culture methods induce

Figure 7. Effect of Gal-9 on high-glucose induced the expression of p27Kip1 and p21Cip1 in MPC and MES. Gal-9 inhibits the protein expression of p27Kip1 and p21Cip1 in a dose-dependent manner, and maximum inhibition effect is observed at a concentration of 1 μM revealed by Western blotting (A). We cultured differentiated MPC and MES. In MPC culture, DMEM that contained 0.5% FCS for 48 h and mature podocytes were incubated with NG, NG+Gal-9, HG, HG+Gal-9, and MN for 6 d. By Western blot analysis, protein levels of p27Kip1 and p21Cip1 increase in HG ambience compared with NG. By densitometric analysis, Gal-9 significantly suppresses p27Kip1 and p21Cip1 (B). High glucose increases the mRNA level of TGF-β1 without statistical significance, and Gal-9 shows no effect on the expression of TGF-β1 mRNA (C). In MES culture, protein levels of p27Kip1 and p21Cip1 increase in HG ambience compared with NG, and treatment with Gal-9 shows an inhibitory effect on the protein expression of p27Kip1 and p21Cip1 without statistically significant differences (B). High glucose significantly increases the mRNA level of TGF-β1, and Gal-9 showed minimal inhibition on the expression of TGF-β1 mRNA (C). Data are mean ± SEM; *P < 0.01 versus NG; **P < 0.01 versus HG.

Figure 8. Effect of Gal-9 on high-glucose–induced G1 cell-cycle arrest of MPC. We cultured differentiated MPC in DMEM that contained 0.5% FCS for 48 h, and mature podocytes were incubated with NG, NG+Gal-9, HG, HG+Gal-9, and MN for 6 d. The cell-cycle distribution of MPC was analyzed by laser scanning cytometer after staining with propidium iodide (A through D). HG increases cells in G0/G1 phase and decreases cells in S and G2 phases compared with cells in NG environment, meaning thereby that G0/G1-phase cell-cycle arrest is observed only in HG ambience. Addition of Gal-9 decreases G0/G1 population and increases cells in S and G2 phases. Data are mean ± SEM; n = 3.
cellular proliferation with the loss of differentiation and thus are not suitable for the investigation of high-glucose–induced G1-phase cell-cycle arrest. However, MPC, conditionally immortalized cells, cultured under successive permissive and nonpermissive conditions usually differentiate into mature podocyte and thus were considered suitable for this investigation. Using MPC on glass slides, we were able to measure total and peak intensity of propidium iodide fluorescence with generation of detailed images of the cell cycle in an unperturbed MPC population by laser scanning cytometry (53). Gal-9 treatment normalized high-glucose–induced G1-phase cell-cycle arrest in MPC. Although nuclear condensation in M phase was observed, no apoptosis in MPC could be observed after Gal-9 treatment. However, Gal-9 dampened MPC hypertrophy because high-glucose–induced de novo protein synthesis was inhibited, leaving DNA replication unaltered. Because TGF-β1 is not expressed abundantly in MPC and not altered by high-glucose condition in our study and previous reports (54), Gal-9 has direct therapeutic effects on cell-cycle arrest in addition to glucose condition in our study and previous reports (54), Gal-9 not expressed abundantly in MPC and not altered by high-glucose–induced de novo protein synthesis of protein, p27Kip1 and p21Cip1, and TGF-β1 expression; however, the effects did not enter the statistically significant differences. This result will not negate the possibility that Gal-9 exerts the therapeutic potential by acting on mesangial cells. We speculated that mesangial cells in culture actively proliferate, and, unlike MPC, the mesangial cells did not well reflect the status of cellular hypertrophy in vivo in diabetic nephropathy.

In previous studies, Gal-9 induced apoptosis in various cells, including thymocytes and activated CD4+ and CD8+ T cells. Gal-9 also acts as eosinophil chemoattractant (10), and it suppresses apoptosis of eosinophil in eosinophilic patients, whereas it enhances apoptosis of eosinophils in normal volunteers (59). In addition to such cells of immune system, recombinant Gal-9 induces apoptosis in human melanoma cell line (MM-RU) (60). It is interesting that in cells of nonimmune origin, the expression of Gal-9 is upregulated by IFN-γ, for instance in human lung fibroblast cell line (61), umbilical vein endothelial cells (62), and WM9 melanoma cells (17). IFN-γ-induced Gal-9 has been implicated to exert an antitumor effect by inducing cell-cycle inhibition and apoptosis of malignant cells, and Gal-9 expression has been inversely correlated with progression of the disease in melanoma (60). Thus, Gal-9 contributes to the elimination of antigen-activated T cells or various malignant cells to maintain proper homeostasis in cells and tissues (4).

We have shown that Gal-9 promotes and assists cell-cycle progression and successful replication in diabetic state, where cell-cycle progression is halted despite cell-cycle entry. Thus, Gal-9 exerts dual action on the cells and modulates the fate of cells, i.e., apoptosis subsequent to S-phase arrest or successful progression to G2 phase depending on the status or the nature of the cells. A similar scenario has been reported with Gal-1. Various cancer cells respond to Gal-1 by entering programmed cell death after S-phase cell-cycle arrest, whereas it causes no significant effect on normal cells and thus acts as a physiologic negative cell-cycle regulator with successful replication of the cells (15). In renal hypertrophy in diabetes, Gal-9 inhibited glomerular TGF-β1 expression and cell-cycle–dependent hypertrophy of the podocytes and mesangial cells by reducing p27Kip1 and p21Cip1 protein levels. We and others have already suggested that the recombinant soluble Gal-9 may have therapeutic potential in various diseases, such as autoimmune or allergic diseases (9) and malignancies (60), in which it conceivably induces selective apoptosis of activated T cells and malignant cells, respectively. From the data of this investigation, one may suggest application of Gal-9 to control cell-cycle–dependent cellular hypertrophy, such as seen in glomerular cells in diabetes, cardiac muscle cells in hypertension, and smooth muscle cells in atherosclerosis. Finally, because Gal-9 recognizes various cell-surface glycoproteins in different cell types and in various cell cycle states, it may worthwhile also to identify such glycoproteins toward which blocking therapeutic interventions can be instituted.

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