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Growth pattern switch of renal cells and expression of cell cycle related proteins at the early stage of diabetic nephropathy

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Abstract

Renal hypertrophy, partly due to cell proliferation and hypertrophy, has been found correlated to renal function deterioration in diabetes mellitus. We screened the up-regulated cell cycle related genes to investigate cell growth and the expression of cell cycle regulating proteins at the early stage of diabetic nephropathy using STZ-induced diabetic rats. Cyclin E, CDK_2 and P^{27} were found significantly up-regulated in diabetic kidney. Increased cell proliferation in the kidney was seen at day 3, peaked at day 5, and returned to normal level at day 30. Cyclin E and CDK_2 expression also peeked at day 5 and P^{27} activity peaked at day 14. These findings indicate that a hyperplastic growth period of renal cells is followed by a hypertrophic growth period at the early stage of diabetes. The growth pattern switch may be regulated by cell cycle regulating proteins, Cyclin E, CDK_2, and P^{27}.

Keywords: Diabetic nephropathy; Proliferation; Hypertrophy; Cyclin E; CDK_2; P^{27}

Diabetic nephropathy (DN) is a common cause of end-stage renal disease, which encompasses a complex of pathological changes, including renal hypertrophy, accumulation of extracellular matrical components, glomerulosclerosis, tubular atrophy and interstitial fibrosis. A correlation has been found between kidney size and renal function deterioration after 8 years of follow-up in patients with type 1 diabetes [1]. Renal hypertrophy is partly due to accumulation of extracellular matrices, as well as renal cell proliferation and hypertrophy [2,3]. Cell proliferation increased in glomeruli and tubulointerstitium in diabetic nephropathy [4,5].

Ultimately, cell growth is controlled by cell cycle regulatory proteins [6]. It is necessary for cyclin dependent kinase (CDK) activation to form complexes with a specific cyclin [7]. Cyclin-kinase inhibitors (CKIs) bind to and inactivate cyclin-CDK complexes and cause cell cycle arrest [8]. CKIs, such as P^{21} [9], P^{27}[10] and P^{57} [11], inhibit cell proliferation by inhibiting cyclin-CDK complexes in the G1 phase and S phase [8]. Cyclins mediate the physical growth of cells and are necessary for both hyperplastic growth and hypertrophic growth. Cyclin E kinase determines whether the growth pattern will be hyperplasia or hypertrophy. If cyclin E is activated, the cell cycle will progress into S phase and complete the remaining phase of the cycle. If it is inhibited, or its activity is not sufficient, the cell cycle will fail to progress into S phase, and therefore, arrest in G1 phase [12]. Increasing evidence shows that cell proliferation and the regulating factors are important for the progress of renal lesions in many kinds of renal diseases. High glucose can cause proliferation in cultured mesangial cells [13], but the detailed events of cell growth and its regulating mechanism are still unclear in diabetic kidneys. We observed

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renal cell growth patterns and the expression of cell cycle-related proteins in STZ-induced diabetic rats to evaluate the possible role of these proteins on the development of diabetic nephropathy.

Materials and methods

Animals. Male Sprague–Dawlay rats weighing 200–300 g were divided into two groups: a control group and a diabetic group (DM group). Animals in the diabetic group received a single intraperitioneal injection of streptozotocin (STZ, Sigma, St. Louis, MO) dissolved in 0.1 mol/L sodium citrate (pH 4.5) at a dose of 65 mg/kg; the control group received the same volume of sodium citrate. Animals were allowed free access to water and standard rat food. In the diabetic group, only those rats with a blood glucose concentration (measured at 11:00 AM) greater than 16.7 mmol/L 48 h after the STZ injection and at the time of sacrifice, along with negative urine ketones, were included in the study.

Tissue preparation. The animals were sacrificed at day 3, 5, 9, 14, and 30 after diabetes induction. Blood glucose levels were measured using a One Touch Basic Glucometer (Qusnsons, USA). Urine samples were collected before sacrifice and 24-h urinary protein excretion was measured. The animals received a single intraperitioneal injection of 100 μg BrdU/g body weight two hours prior to sacrifice. The kidneys were removed and weighed. The specimens were partially fixed in 4% paraformaldehyde for light microscopy and immunohistochemistry. Specimens were also partially fixed in 70% ethanol for flow cytometry. Kidneys of three animals in the DM group were collected at day 9 for RNA extraction.

Gene chip assay. Total RNA was extracted with one-step method and the mRNA was purified with Oligo-dT cellulose. The fluorescent cDNA probes were prepared through reverse transcription. The RNA samples from the control group were labeled with Cy3-dUTP and those from the DM group with Cy5-dUTP. The hybridized microarrays consisting of 324 cell cycle-related cDNAs were scanned with a ScanArray 3000 (GSI Lumonics, Billerica, MA, USA) at two wavelengths to detect emission from both Cy3 and Cy5. The acquired images were analyzed using ImaGene 3.0 software (BioDiscovery, Inc., Los Angeles, CA, USA). The threshold value to define significant relative expression changes was set at 2.0 for over-expression and at 0.50 for under-expression, based on both the experimental variability in our data and the manufacturer's established performance criteria.

Light microscopy and immunohistochemistry. The renal tissues fixed in 4% paraformaldehyde were embedded in paraffin and cut into sections of 4μm thickness. The sections were stained with HE and SP immunohistochemical method. Antibodies used included mice anti-rat BrdU monoclonal antibodies (Calbiochem Inc, USA) and rabbit anti-rat antibodies against Cyclin E, CDK_2 and P^{27} (Santa Cruz). After deparaffinization and rehydration, the tissue was incubated with 3% H_2O_2 for 15 min at room temperature to block any intrinsic peroxidase activity. The tissue was also incubated with 20% normal goat serum for 2 h at 37°C to prevent non-specific adherence of serum proteins. The tissues were then incubated sequentially with anti-rat antibodies (1:50) for 1 h at 37°C, biotinylated anti-rabbit IgG (1:100; Gibco-BRL) for 20 min and streptavidin-peroxidase conjugate for 20 min. The number of cells and diameter of glomeruli and tubules were quantitatively analyzed with the TD 2000 image pattern analysis system. Fifty glomeruli and 100 tubules for each animal were evaluated.

Flow cytometry. The renal tissues fixed in 70% ethanol were used to prepare single cell suspensions. The cells (1×10^4) were resuspended in PBS with 0.1% bovine serum albumin and were incubated for 30 min at 4°C with antibodies against Cyclin E, CDK_2 and P^{27} (1:100), which was followed by an incubation for 30 min at 4°C with FITC-conjugated goat anti-rabbit IgG. The cells were then analyzed on flow cytometry (FACS 420, BD Company, USA). At least 10,000 events were collected for each sample, and the data displayed on a logarithmic scale of increasing green-fluorescence intensity. A fluorescence index (FI) was used to evaluate the expression of Cyclin E, CDK_2 and P^{27}.

Western blot. Twenty micrograms of protein from each sample was subjected to SDS/PAGE under reducing conditions and the gel proteins were electroblotted on to Hybond P PVDF membrane (Amersham). Membranes were incubated with rabbit polyclonal anti-Cyclin E, CDK_2 and P^{27} antibodies (1:500) and anti-rabbit IgG conjugated with horseradish peroxidase (1:20,000) at 37 °C. The blots were incubated with ECL® Plus Western Blotting Detection Reagents (Amersham), and then exposed to X-ray film [14].

Immunoprecipitation kinase assay. Two hundred micrograms of protein from each sample was suspended in 1.0 ml of kinase lysis buffer (50 mM Hepes [pH 7.6], 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 0.1 mM $NaVO_3$, 1 mM NaF, 0.1% Tween 20, 10% glycerol, 1 mM DTT, 1 mM PMSF, and 4 μg/ml leupeptin) and precleared by incubation with normal IgG (1 μg) and protein A-Sepharose (Sigma) for 1 h at 4 °C. The supernatant was collected and incubated with 1.0 μg of the rabbit polyclonal anti-CDK2 antibody (Santa Cruz) and 20 μl of protein A-Sepharose overnight at 4 °C. The immunoprecipitants were washed three times with the kinase lysis buffer. The beads were then incubated with 40 μl of kinase solution containing 5.0 μg of histone H1, 80 μM ATP, and 5 μCi of [γ-^{32}p]ATP (6000 Ci/mmol) in kinase buffer (40 mM Tris–Cl [pH 7.6], 20 mM $MgCl_2$, 2 mM DTT) for 30 min at 37 °C. The samples were resolved over a 12% polyacrylamide gel. The gels were dried using a Bio-Rad gel drier followed by detection using autoradiography with X-ray film.

Statistical analysis. The data were analyzed with Dr. SPSS II for Windows release 11.0.1 J. Statistical significance was defined as $P < 0.05$, and all values were expressed as means ± SD.

Results and disscusion

Diabetic nephropathy is a common cause of diffuse glomerulosclerosis and renal failure. It was reported that renal hypertrophy at the early stage of DN was related to the development of glomerulosclerosis at the advanced stage [2]. Glomerular and tubular hypertrophy are the early structural alterations found in diabetic nephropathy [15–17]. However, the cellular events of the kidney as well as the regulating mechanism of renal cell growth at the early stage of DN are not clear.

Here, we used STZ-induced diabetic rats to investigate the cellular events at the early stage of diabetic nephropathy. It was reported that early transplantation of pancreatic islets prevented the development of renal hypertrophy in STZ-induced diabetic rats [18]. We also confirmed that the renal hypertrophy in diabetic kidney was normalized by insulin treatment (data not shown). Thus we conclude that the nephropathy was caused by a diabetic state other than STZ itself, and the dose of STZ for diabetes induction was not high enough to cause nephropathy.

We found that at the very early stage of diabetic nephropathy, the kidney underwent hypertrophy three days after STZ induction and the kidney/body weight ratio in the DM group increased significantly at day 5. The weight ratio was maximal at day 14 and remained at this level on day 30 (Fig. 1A). This indicates that the kidney enlarged within days after the STZ injection. Young et al. reported that the number of proliferating cell nuclear antigen (PCNA) positive cells increased and peaked at day 3 after STZ injection in the renal cortex of diabetic rats, then decreased slowly [19]. However, in another study, the increase of PCNA positive cells was not observed at day

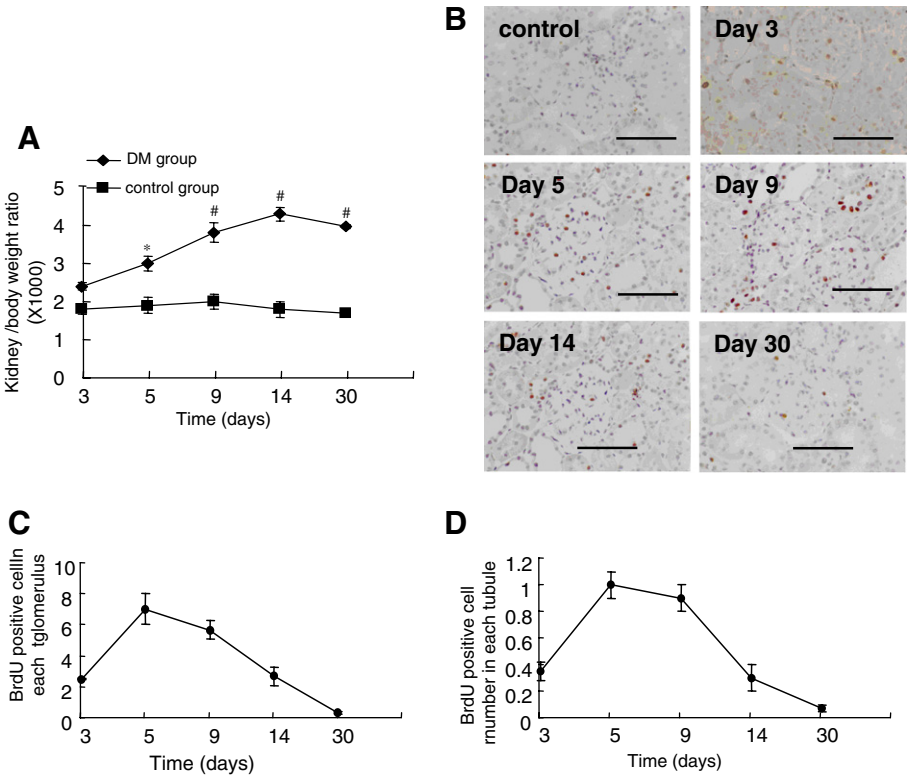

Fig. 1. Kidney/body weight ratio and BrdU positive cells in kidney. (A) Kidney/body weight ratio. Compare with that in control group, the kidney/body weight ratio in diabetic group increased from day 5. (B) Immunohistochemical staining for BrdU incorporation. BrdU positive cells were rarely seen in the renal cortex of control group and significantly increased in DM group at day 3, peaked at day 5, then decreased gradually, and recovered to a baseline as control group at day 30(C, D). Scale bars, 100 μm (B). $^{*}p < 0.05$, $^{\#}p < 0.01$ vs control group. $N = 6$ rat per group.

3 and day 9 after STZ injection, although glomerular volume and kidney/body weight ratio increased [20]. To determine whether hyperplasia contributes to renal hypertrophy, we used BrdU as a marker of proliferation [21,22]. As shown in Fig. 1B–D, BrdU positive cells are rarely seen in a normal control kidney, whereas, either in glomerli or in tubules, the BrdU positive cells increased at day 3, peaked at day 5, and decreased to control levels at day 30. These results were consistent with the changes of glomerular and tubular cell numbers shown in Fig. 2, where the number of glomerular cells increased from day 5, maximized at day 9, and deceased slightly at day 30; the number of tubular cells increased from day 3, then experienced similar changes as the glomerular cells. From day 9, the glomerular diameter and the tubular diameters increased gradually. The results suggested that, in diabetic rats, renal cell growth underwent an initial hyperplastic period, followed by a hypertrophic period. How was the cell growth was regulated? The mechanism of the pattern switch is not clear.

The cell cycle is controlled by cell cycle regulatory proteins, such as cyclins, cyclin-dependent kinase (CDK) and cyclin-kinase inhibitors (CKIs)[16]. In mammalian cells, cyclins form active protein kinase complexes with CDK proteins, which are required for progression of cells through G1 into S phase [23]. During S phase, the DNA strands are separated, stabilized, and then DNA is replicated by polymerases. Here we screened the cell cycle-related proteins in diabetic kidneys by gene chip assay. Among the 324 cell cycle-related cDNAs, there were a total of 12 genes up-regulated in DM group whose cy3/cy5 ratio were over 2, the top three are cyclin E (cy3/cy5: 5.6), CDK_2 (cy3/cy5: 6.1) and P^{27} (cy3/cy5: 4.8).

To examine the mechanism of how these cell cycle-related proteins are responsible for the growth pattern switch, we evaluated their expression levels at day 3, day 5, day 9, day 14 and day 30 using flow cytometry. The results were confirmed by Western blot. As shown in Fig. 3, the increases in expressions of both cyclin E and CDK_2 were detected at day 3 and peaked at day 5. The increased cyclin E and CDK_2 expression were consistent with the increase of BrdU incorporation due to the enhanced G1-S transition at these time points. The expression of CDK_2 remained at a high level, but the expression of cyclin E returned to the control level at day 14 when the significant increase of P^{27} expression was detected. The recovery of cyclin E activity and increase of P^{27} activity was also shown as the P^{27}/cyclin E ratio (see Fig. 3D), remained consistent with the decrease of BrdU incorporation. The P^{27}/cyclin E ratio increased at day 9 and peaked at day 14, which suggested the arrest of the G1–S transition, a key characteristic of cell hypertrophy [15]. From the results of the radioactive kinase activity assay shown

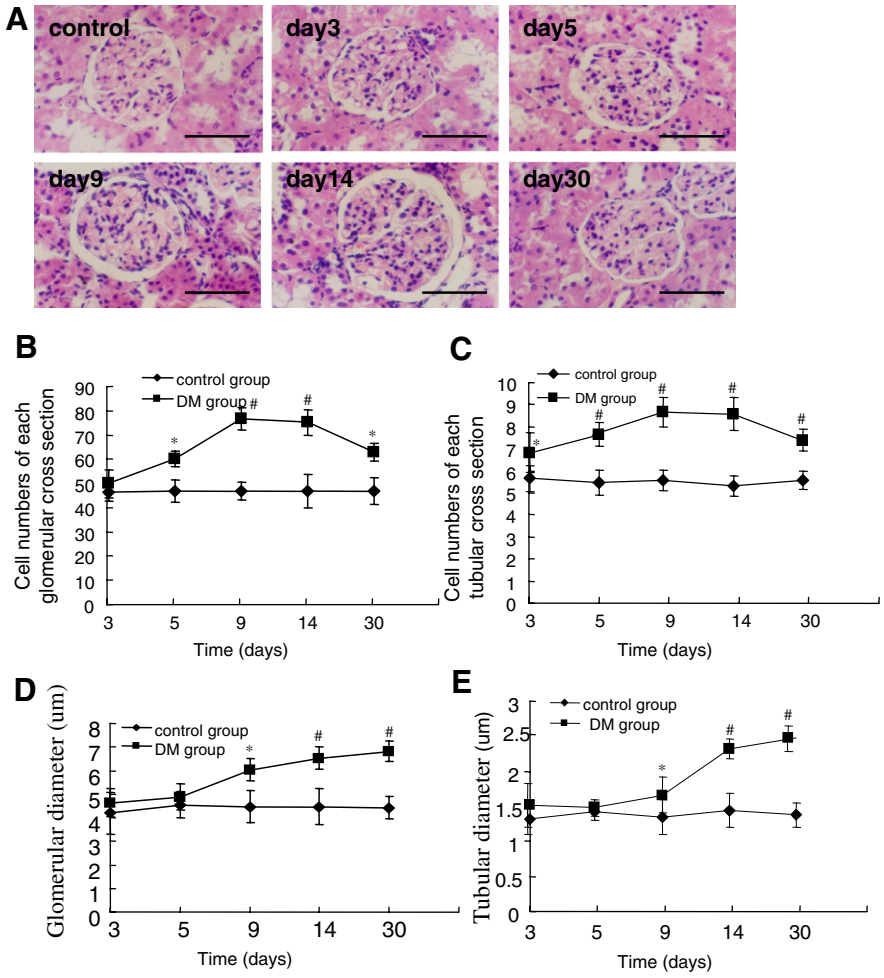

Fig. 2. Cell numbers and diameters of each glomerular and tubular cross section in control and diabetic kidney. (A) Representative sections of HE staining. Cell numbers of glomeruli (B) increased from day 5 and that of tubules (C) increased from day 3; Glomerular and tubular diameters increased from day 9 (D,E). ($^{*}p < 0.05$, $^{\#}p < 0.01$ vs control group). Scale bars, 100 μm (A). $N = 6$ rat per group.

in Fig. 4, comparing with the normal control rats, the diabetic rats showed increased CDK_2 kinase activity in the kidney at day 3; the kinase activity peaked at day 5, and maintained at a higher level until day 14. It then decreased to the normal control level at day 30, which is consistent with the increase of P^{27} activity. Kinase activity of the cyclin/CDK complexes is negatively regulated by CKIs. An overall increase in CKIs directly inactivates cyclin/CDK complexes by binding to them and interfering with their kinase activity. Thus, it makes the cells arrest at G1 phase and undergo hypertrophy [23]. From these results, we conclude that at day 14, renal cell growth patterns switch from proliferation to hypertrophy. P^{21} and P^{57} are the other two CKIs that can bind to and inhibit the kinase activity of cyclin E/CDK_2 and halt the progression of the cell cycle in G1 phase [9,11]. We also tested p^{21} and p^{57} expression by flow cytometry and Western blot. A slight increase of P^{21} and P^{57} was seen at day 14, but no statistical significance was observed between the control group and DM group (data not shown). From the above results, we conclude that the cell cycle progression at the early stage of diabetic nephropathy is mainly regulated by cyclin E, CDK_2, and P^{27}.

The present results suggest that in diabetic rats, renal cell growth underwent an initial hyperplastic period, followed by a hypertrophic period. Cell cycle-related proteins, cyclin E, CDK_2 and P^{27}, may play important roles for the switch from hyperplasia to hypertrophy. Renal hypertrophy is an early abnormality of diabetic nephropathy. Accumulating evidences suggest that this early hypertrophy may act as a pacemaker for the subsequent irreversible structural changes such as glomerulosclerosis and tubulointerstitial fibrosis [23]. Megyesi [24] reported that littermates with a homozygous deletion of the gene for the cyclin dependent kinase inhibitor, P^{21}, do not develop chronic renal failure after partial renal ablation. According to these results, Awqati and Preisig [25] believed that knockout of P^{21} may save the kidney by limiting compensatory renal growth. It is imaginable that prevention of renal hypertrophy by modifying cell cycle-related proteins, such as Cyclin E, CDK_2 and P^{27}, may prevent progression of diabetic nephropathy by normalizing the cell growth events in

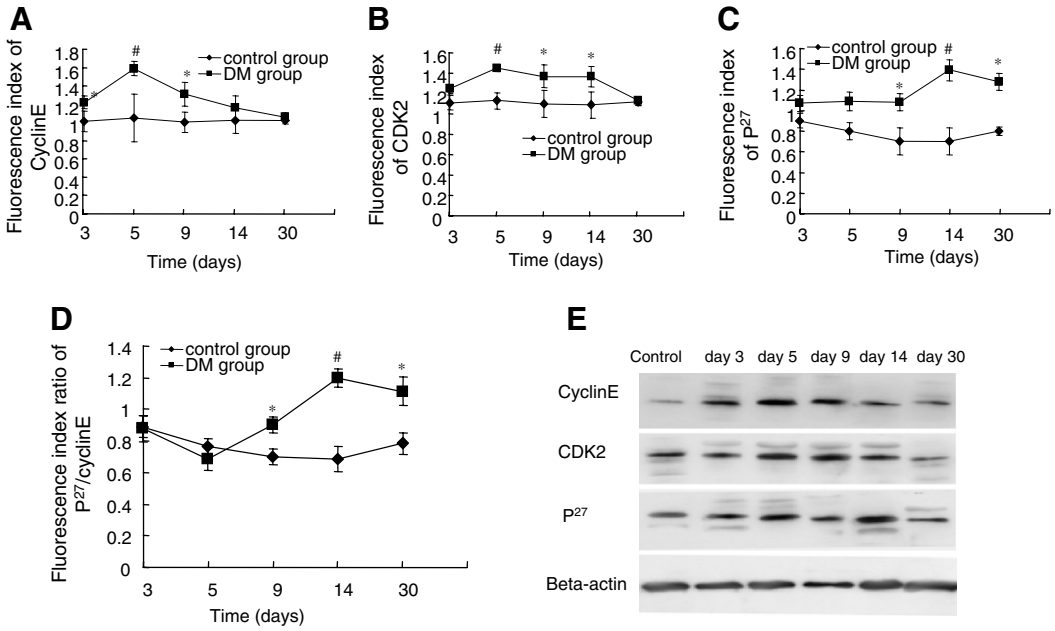

Fig. 3. Cell cycle related proteins, cyclin E, CKD2 and P^{27} expression. From the results of flow cytometry, cyclin E and CDK_2 were increased at day 3 and peaked at day 5, P^{27} expression peaked at day 14 (A–C); The ratio of P^{27}/cyclin E was increased at day 9 and peaked at day 14 (D); The results were confirmed by Western blot using renal cortex proteins (E). $N = 6$ rat per group.

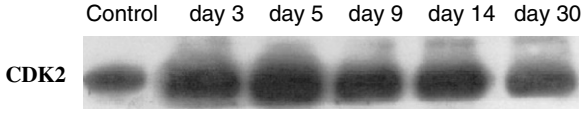

Fig. 4. Kinase activity associated with CDK_2. The CDK_2 kinase activity was comparatively low in normal control kidney (control); after diabetes induction, the kinase activity increased at day 3, peaked at day 5 and maintained at a a higher level until day 14, and then decreased to the control level at day 30.

diabetes mellitus. Thus Cyclin E, CDK_2 and P^{27} might serve as novel targets in the therapeutic interventions for diabetic nephropathy.

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