The Expression of Intermediate Filament Protein Nestin and Its Association With Cyclin-dependent Kinase 5 in the Glomeruli of Rats With Diabetic Nephropathy

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Abstract: *Background:* Podocyte injury plays a crucial role in the development of diabetic nephropathy (DN), but its underlying mechanism remains poorly understood. Emerging evidences suggest that the cytoskeleton disruption is related to podocyte injury. The aim of this study was to investigate whether nestin, a cytoskeleton-associated intermediate filament protein, is involved in the development of DN. *Methods:* Rat diabetes was induced by intraperitoneal injection of streptozotocin. The renal histological changes were investigated by light microscopy and transmission electron microscopy. The location of nestin and vimentin in renal tissues was observed by immunohistochemistry. The protein or messenger RNA levels of nestin and cyclin-dependent kinase 5 (Cdk5) were detected by Western blot and real-time polymerase chain reaction. The relationship between nestin and vimentin was detected by co-immunoprecipitation. *Results:* Compared with controls, diabetic rats showed significant characteristics of renal damage. The expression of nestin and vimentin in the glomeruli was increased at the early stage of diabetes, which then gradually decreased. Co-immunoprecipitation assays demonstrated that nestin disassembled with vimentin in diabetic rats. The expression of Cdk5 was increased in a time-dependent manner in diabetic rats. The degree of albuminuria in diabetic rats was negatively correlated with nestin and positively correlated with Cdk5. Roscovitine, a Cdk5 inhibitor, reduced the degradation of nestin. Moreover, podocyte injuries were significantly ameliorated by treatment with roscovitine. *Conclusions:* The intermediate filament protein nestin is associated with development of DN. Blockage of Cdk5 increases the level of nestin and attenuates renal damage, which would provide a useful target for DN therapy.

Key Indexing Terms: Nestin; Cyclin-dependent kinase 5; Diabetic nephropathy; Roscovitine; Vimentin. **[Am J Med Sci 2013;345(6):470–477.]**

Diabetic nephropathy (DN) is one of the most common complications in diabetic patients and has been a major public health problem in modern society. Podocytes, terminally differentiated and highly specialized cells, play an indispensable role as a filtration barrier for macromolecules in the glomerulus. Podocyte injuries, including foot process effacement, hypertrophy, detachment and perhaps epithelial-to-mesenchymal transition, which are considered to be important in terms of causing albuminuria and glomerular damage, play a critical role in the development of many glomerular diseases including DN.[1–4]

However, the underlying mechanisms of podocyte injury in DN remain poorly understood. Recently, several studies have shown that the cytoskeleton disruption is related to podocyte injury.[5–7] Nestin is a cytoskeleton-associated class VI intermediate filament (IF) protein, which was first described as a marker for neuroepithelial stem cells.[8] Subsequently, nestin was also identified in progenitor cells from various non-neuronal tissues.[9–12] Recently, nestin has been reported to be stably expressed in the podocytes of mature glomeruli in adult kidney,[13,14] and plays an important role in maintaining the normal morphology and function of podocytes. In cultured mouse podocytes, the knockdown of nestin by small interfering RNA significantly reduced the number of podocyte processes.[13] From clinical studies, it was shown that, compared with normal subjects and patients without proteinuria, nestin expression of glomerular podocytes in nephropathic patients with proteinuria was significantly reduced, and improvement in proteinuria after medical management was accompanied by an increase in nestin expression in those patients.[15] However, little is known about whether the cytoskeleton protein is involved in the diabetic glomerular damage, and the changes of nestin during the development of DN have not yet been established.

Cyclin-dependent kinase 5 (Cdk5) is well known as a key regulator of neuronal development and normal neuronal function.[16] Several studies have demonstrated that nestin is a substrate for Cdk5,[17] and inhibition of Cdk5 activity can reduce nestin degradation, indicating that Cdk5 is involved in the regulation of nestin turnover.[18] Within the kidney, the expression of Cdk5 is limited to podocytes, and it also regulates the morphology of podocytes. However, under a high-glucose environment, the effects of Cdk5 on nestin expression and podocyte injury have never been documented. To determine the potential significance of nestin and Cdk5 expression in diabetic nephropathy, we examined the expression levels of nestin and Cdk5 in the glomeruli of diabetic rats, and also detected the effects of Cdk5 blockage with roscovitine on nestin expression and renal damage.

MATERIALS AND METHODS

Animals and Experimental Design

Male Wistar rats (200–220 g) were obtained from the Experimental Animal Center of the Hebei Medical University (Shijiazhuang, Hebei, China). All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee of the Hebei Medical University. After 7 days of acclimatization, Wistar rats were randomly divided into 4 groups: normal control group, diabetic group, diabetes with roscovitine treatment group and diabetes with dimethyl sulfoxide (DMSO) treatment group. Type 1 diabetes mellitus

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was induced by intraperitoneal injection of streptozotocin (STZ; 65 mg/kg; Sigma Chemicals, St. Louis, MO) dissolved in 0.1 M citrate buffer (pH 4.5). Individual animals with blood glucose concentrations >16.7 mM for 3 consecutive days were confirmed as diabetic. The control rats received 0.1 M citrate buffer solution only. After confirmation, diabetic rats of the roscovitine treatment group were given an intraperitoneal injection of roscovitine daily (Sigma Chemicals), at a concentration of 2.8 mg/kg body weight dissolved in a total volume of 400 μL DMSO,[19] whereas those in the DMSO treatment group were given an intraperitoneal injection of 400 μL DMSO as control.

Rats were housed in a temperature-controlled room and were given free access to water and standard laboratory chow during the study period. At 4, 8, 12 and 16 weeks after treatment, 6 rats from each group were weighed and housed individually for 24 hours in metabolic cages for collecting urine, to measure 24-hour urinary albumin or proteins (on a Siemens Immulite 1000 chemistry analyzer; Siemens, Munich, Germany). Their sera were prepared for the measurement of serum blood glucose and blood urea nitrogen (BUN) concentrations. Then, the rats were killed and their kidneys removed. One portion of the renal tissues was fixed in 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) for histological and immunohistochemical examinations, and another portion was fixed immediately in 4% glutaraldehyde for transmission electron microscopy. The remaining renal tissues were prepared for isolating the glomeruli.

Histopathologic Examination

The paraffin-embedded renal tissue sections (2 μm) were stained with periodic acid-Schiff staining and examined under the light microscope. The glutaraldehyde-fixed renal tissues were further postfixed with 1.5% osmic acid and embedded in Quetol 812 mixture (Nissin, Tokyo, Japan). The ultrathin sections were stained with uranyl acetate and lead citrate, and examined under a Hitachi H7500 transmission electron microscope (Hitachi, Tokyo, Japan).

Immunohistochemistry

The expression of nestin in the glomeruli of kidney was characterized by immunohistochemistry using mouse anti-nestin antibody (Abcam, Cambridge, United Kingdom) and rabbit anti-vimentin antibody (Proteintech, Chicago, IL). Three- to 5-μm sections were cut from each specimen, mounted on electrostatically charged slides and dried. Sections were dewaxed, rehydrated and washed in 0.01 M PBS. Antigenic sites were unmasked by heating sections in 0.01 M citrate buffer (pH 6.0) in a microwave oven and were subsequently rinsed for 15 minutes in 3% H_2O_2 to block endogenous peroxidase. Slides were then incubated with anti-nestin antibody (1:200) or anti-vimentin antibody (1:200) at 4°C overnight. The following day, the sections were washed with PBS, and then incubated with the PV-9000 Polymer Detection System (Zhongshan Golden Bridge Biotechnology, Beijing, China). The antibody was detected with diaminobenzidine, and the sections were counterstained with hematoxylin. Negative controls were obtained by replacing the specific antibody with PBS. The sections were imaged using the Olympus microscope and characterized quantitatively by digital image analysis using the Image Pro-Plus 5.0 image analysis software (Media Cybernetics, Silver Spring, MD).

Glomerular Isolation

Kidneys were excised from the rats and placed in sterile Hanks' balanced salt solution. The renal cortices were minced and pressed onto a sterile stainless sieve, and passed through serial sterile sieves of various pore sizes as described previously.[20,21] On the sieve No. 200 (pore size, 75 μm), glomeruli were retained almost exclusively (<5% tubular fragments). Glomeruli on the sieve were collected, rinsed with Hanks' balanced salt solution and centrifuged at 435*g* for 10 minutes. The pellets were collected and used for extracting protein and RNA.

Western Blot Analysis

The isolated glomeruli protein extracts (100 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA). The membranes were blocked for 1 hour at 37°C with 5% skimmed milk in Tris-buffered saline containing 0.05% Tween 20 and incubated overnight at 4°C with anti-nestin (1:1000; Abcam), Cdk5 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (1:1000; Santa Cruz Biotechnology) antibodies. After washing 3 times with Tris-buffered saline containing 0.05% Tween 20, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) at room temperature for 2 hours, followed by detection using enhanced chemiluminescence (Pierce, Rockford, IL). Immunoreactive bands were visualized using x-ray films. β-Actin was used as an internal control and the intensity of the bands was measured using LabWorks 4.5.

Co-immunoprecipitation

Immunoprecipitation experiments were carried out on total homogenates of the renal glomeruli as previously described.[14] Isolated glomeruli were placed in homogenization buffer (50 mM Tris/HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.25% sodium deoxycholate, 1% NP-40, 1 mM phenylmethyl sulfonyl fluoride, 2 mg/mL aprotinin, pH 7.4) and homogenized. The suspension was centrifuged at 5000*g* for 10 minutes at 4°C, and the protein content was measured with the Coomassie Protein Assay Kit (Pierce). Two hundred micrograms of proteins of each group was precleared with 20 μL protein A Sepharose (Santa Cruz Biotechnology) at 4°C for 3 hours. Supernatants were collected and 2 μg of anti-nestin antibody was added per sample, and then incubated for 2 hours at 4°C with constant rotation. Immunoprecipitation was performed by the addition of 25 μL protein A Sepharose to each tube and incubated with rotation at 4°C overnight. Conjugated beads were collected by centrifugation and washed 3 times with homogenization buffer before addition of 25 μL of 2× loading buffer and heating at 95°C to 100°C for 5 minutes. Associated proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane, followed by Western blot analysis with anti-vimentin antibody (1:1000; Santa Cruz Biotechnology). The intensity of the individual bands was measured using LabWorks 4.5.

Quantified Real-Time Polymerase Chain Reaction

Total RNA was extracted using the TRIzol Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized from the total RNA (0.5 μg) using the PrimeScript RT regent kit following the instructions provided by the manufacturer (Takara Biotechnology, Dalian, China). Subsequently, the complementary DNA was subjected to real-time polymerase chain reaction (PCR) using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, United Kingdom). Each real-time PCR reaction consisted of 2 μL of diluted reverse transcriptase product, 10 μL of SYBR Green PCR Master Mix (2×) and 250 nM forward and reverse primers in a total volume of 20 μL. Reactions were carried out on 7500 Real-Time PCR System