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Downregulation of CD2-associated protein impaired the physiological functions of podocytes

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Abstract

Emerging evidences show that CD2-associated protein (CD2AP) is involved in podocyte injury and the pathogenesis of proteinuria. However, the exact molecular mechanism by which CD2AP exerts its biological function is elusive. We knocked down CD2AP gene by target siRNA in conditionally immortalized mouse podocytes, which showed lowered cell adhesion and spreading ability ($P < 0.05$). At the same time, cell cycle was arrested in G2/M phase ($P < 0.05$), and pathologic nuclear division could easily be seen in CD2AP siRNA-transfected podocytes. The proliferation of podocytes were also inhibited significantly by CD2AP siRNA transfection ($P < 0.05$). Further study revealed disordered distributions of F-actin, as well as lowered nephrin expression and phosphorylation in podocytes. These data suggest that CD2AP may play a crucial role in maintaining the normal function of podocytes and lowered CD2AP causes podocyte injury by disrupting the cytoskeleton and disturbing the nephrin-CD2AP signaling pathway.

Keywords: CD2AP; Podocytes; Cytoskeleton; Nephrin

1. Introduction

Podocytes are highly differentiated epithelial cells covering the outermost layer of glomerular filtration barrier. With respect to their architecture, podocytes consist of 3 segments: cell body, major process and foot process (Asanuma and Mundel, 2003). The foot processes of adjacent podocytes are regularly interdigitated to form the slit diagram (SD) and prevent plasma proteins from leaking into urine (Reiser et al., 2000). In the past 10 years many molecules have been revealed to contribute to the composition of SD structure, such as nephrin, podocin, Neph-1, FAT, CD2-associated protein (CD2AP) and α-actinin 4 (Shih et al., 1999; Tryggvason, 1999; Donoviel et al., 2001; Inoue et al., 2001). Genetic studies have identified that mutation of these genes, which encode respective SD proteins, are associated with the development of massive proteinuria in congential or acquired nephrotic syndrome (Gubler, 2003; Garg et al.,2007).

CD2AP is originally regarded as an adapter protein that interacts with the cytoplasmic domain of CD2, a T cell and natural killer cell membrane protein, and facilitates T cell adhesion to antigen-presenting cells (Dustin et al., 1998). In kidneys CD2AP expresses predominantly in podocytes. Early studies of CD2AP (−/−) mice, which die of massive proteinuria shortly after birth, have suggested the pivotal role of CD2AP in maintaining the integrity of glomerular filtration barrier (Shih et al., 1999). Another study revealed that CD2AP expression was lowered in many types of glomerulonephritis (Kim et al., 2003), which suggested an important role of CD2AP in podocyte injury and the pathogenesis of proteinuria.

Recently, it was identified that CD2AP binds with p130Cas, a cytoplasmic protein involved in the formation of focal adhesions induced by integrin signaling (Welsch et al., 2001; Seidler et al.,

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2005). CD2AP also interacts with the proto-oncogene product c-Cbl involved in tyrosine kinase signaling and regulation of lamellipodia formation and cell morphology (Scaife and Langdon, 2000). CD2AP is also concentrated in the narrow region of the midzone microtubules in the cytokinesis process of Hela cells (Monzo et al., 2005). This suggests that CD2AP play an important role in maintaining cytoskeleton and cell morphology. However, the exact molecular mechanism by which CD2AP exerts its biological function in podocytes has not been fully elucidated.

To explore the role of CD2AP in maintaining the biological functions of podocytes, we observed the effects of knocking-down CD2AP on podocytes, especially on cell adhesion, spreading, cell cycle, proliferation, and cell division. We also explored the possible mechanism mediating the above effects.

2. Materials and methods

2.1. Cell culture

The conditionally immortalized mouse podocyte cell line generated from kidneys of adult transgenic H-2K^b-tsA58 mice (Mundel et al., 1997), was a gift of Prof. Peter Mundel (Department of Medicine, Mount Sinai School of Medicine, New York, USA). Cells were grown on collagen-I coated flasks or plates in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10–50 units/ml recombinant mouse interferon-γ, 100 U/ml penicillin and 100 mg/ml streptomycin at 33 °C (the permissive condition) or 37 °C without recombinant mouse γ-IFN (non-permissive conditions).

2.2. CD2AP siRNA transfection

Subconfluent podocytes cultured in 33 °C permissive conditions were used for transfection with Metafectene (Biontex Munich, Germany), as indicated by the manufacturer. Briefly, 8 μl of 10 μM CD2AP siRNA control siRNA (Santa Cruz, USA) was diluted into each 35 mm dish with 100 μl of Opti-MEM I (Invitrogen, USA) and incubated for 5 min. In parallel, 10 μl of Metafectene was diluted in 100 μl of Opti-MEM I. Diluted Metafectene and siRNA were mixed and incubated at room temperature for 20 min. The medium was replaced with 500 μl RPMI 1640 serum-free medium and antibiotics, and the siRNA/Metafectene mixture was added into the cells. After replacing the transfection mixture after 6 h with normal RPMI 1640 medium, images were taken using fluorescence phase-contrast microscopy (Nikon ECLIPSE TE2000, Nikon, Japan). Transfection efficiency was evaluated through control siRNA tagged with reporting fluorescence molecule (Fluorescein Conjugate-A), using fluorescence microscopy and flow cytometry after 24 h. The inhibition effect of CD2AP siRNA was also confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot after 48 h.

2.3. Semi-quantitative RT-PCR

Total RNA was extracted from podocytes with TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA samples were quantified by measurement of optic absorbance at 260 nm and 280 nm in a spectrophotometer, with the A260/A280 ratio ranging from 1.8 to 2.0, which indicated a high purity of the extracted RNA. The concentration of total RNA was calculated according to A260. Aliquots of total RNA (2.0 μg) from each sample were reverse-transcribed into cDNA using First Strand cDNA Synthesis Kit manufacturer (MBI, Lithuania). Equal amounts of the reverse transcriptional products were subjected to PCR amplification. We co-amplified the housekeeping gene GAPDH to allow semi-quantitative comparison of PCR products. All PCR primers were synthesized by Sangon Biotech, China. The primer sequences and the amplified lengths are as the following: CD2AP forward: 5′-CGA GTT GGG GAA ATC ATC AG-3′, CD2AP reverse: 5′-TGA GGT AGG GCC AGT CAA AG-3′ (504 bp); GAPDH forward: 5′-CAT CAC CAT CTT CCA GGA GCG-3′, GAPDH reverse: 5′-GAG GGG CCA TCC ACA GTC TTC-3′ (357 bp). Amplification was started with 4 min of denaturation at 94 °C followed by 34 cycles (for GAPDH, 30 cycles). Each cycle consists of 30 s at 94 °C, 45 s at 56 °C, 60 s at 72 °C. The final extension was for 8 min at 72 °C. After amplification, 5.0 ml of each PCR reaction product was electrophoresed through a 1.5% (w/v) agarose gel containing ethidium bromide (0.5 mg/ml). Gels were scanned using an EPSON GT-800 Scanner (EPSON, Japan) and photos were taken. The mRNA levels of CD2AP were normalized with GAPDH mRNA levels.

2.4. Cell adhesion and spreading assays

96-Well plates were coated with 5 mg/ml collagen IV (Sigma–Aldrich) according to protocols of the manufacturer. CD2AP siRNA-transfected podocytes were seeded into the coated plates at 1.0×10^4 cells/well. The plates were incubated at 33 °C for 90 min. For adhesion measurement, cells were photographed with phase-contrast microscopy (Nikon ECLIPSE TS100, Japan), trypsinized, and counted by flow cytometry. For spreading measurement, adherent cells were fixed with 95% (v/v) alcohol for 15 min and stained with hematoxylin and eosin (HE). Random fields from hexaplicate samples were photographed, and the surface area of 100 cells from each experiment was measured using HPIAS-1000 Image Analysis System (Qingping, China).

2.5. Cell cycle analysis

Cells were seeded in 6-well plates and transfected with CD2AP control siRNA. Three days later, cells were harvested, fixed in 70% ethanol and treated with RNase A. They were incubated in PBS containing 40 μg/ml propidium iodide and analyzed in flow cytometry. CellQuest software was used to analyze cell populations in G_0/G_1, S and G_2/M phases.

2.6. Fluorescence dyeing and microscopy

F-actin microfilament and microtubule changes in podocyte cells were followed after a shift into 37 °C non-permissive condition to induce foot processes formation. Cells were grown on 6-well plates containing glass coverslips in 37 °C for 7 d at low density and fixed in ice-cold acetone in −20 °C for 10 min.