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Angiopoietin-like protein 3 regulates the motility and permeability of podocytes by altering nephrin expression *in vitro*

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ABSTRACT

It is well known that podocyte injury plays a vital role in massive proteinuria. The increase of podocyte motility results in podocyte foot process (FP) effacement, a typical form of podocyte injury. Our previous studies demonstrated that glomerular podocytes can express angiopoietin-like protein 3 (ANGPTL3) and that the increase of ANGPTL3 in dysfunctional glomerulus is correlated with podocyte FP effacement. Little is known, however, about the role of ANGPTL3 in podocyte injury. In this study, we investigated ANGPTL3's effect on the motility and permeability of podocytes and on the expression of nephrin, a key molecule in podocytes. By scrape-wound and transwell migration assay, we found that ANGPTL3 over-expression significantly increased podocyte motility, whereas after ANGPTL3 knockdown by RNA interference, motility remained the same as that of the control group. Adriamycin (ADR) treatment significantly promoted podocyte motility. However, the same dose of ADR treatment could not promote motility after the knockdown of ANGPTL3. In addition, we assayed the diffusion of FITC-BSA across the podocytes' monolayer to investigate whether ANGPTL3 could promote protein loss by means of an increase in podocyte motility. The results showed that the changes in the FITC-BSA permeability of the podocytes corresponded to changes in motility. Furthermore, we found that ANGPTL3 over-expression dramatically increased the expression of nephrin but that the up-regulation of nephrin induced by ADR was significantly inhibited when ANGPTL3 was diminished by RNAi. In conclusion, we found ANGPTL3 to be capable of regulating the motility and permeability of podocytes and that the mechanism of ANGPTL3's regulation could be associated with the altered expression of nephrin.

1. Introduction

Podocytes cover the outer aspect of the glomerular basement membrane (GBM). Thus, they form the final barrier to protein loss, which explains why podocyte injury is typically associated with marked proteinuria [1]. The mechanism of podocyte injury, however, is not well understood. Recent studies have reported that the kidney glomerulus could express angiopoietins (Angs) such as angiopoietin 1 (Ang1) and angiopoietin 2 (Ang2). Further studies demonstrated that Ang1 and Ang2 were involved in podocyte injury [2,3].

Recently, a family of proteins structurally similar to the angiopoietins was identified and designated "angiopoietin-like proteins" (ANGPTLs). As a new member of this family, angiopoietin-like protein 3 (ANGPTL3) has the same structure as the C-terminal fibrinogen (FBN)-like domain (FLD) and N-terminal coiled-coil domain (CLD), among others. According to previous reports, ANGPTL3 was mainly expressed in liver cells and was only weakly expressed in the kidney [4]. In different regions, ANGPTL3 exhibited varying functions, including C-terminal FLD-induced angiogenesis when bound to integrin αVβ3 and increased plasma triglyceride levels in mice when bound to the N-terminal CLD [5,6]. Because of its powerful inhibition of lipoprotein lipase activity, ANGPTL3 is thought to play an important role in lipid metabolism [4,5,7,8]. To date, however, the relationship between ANGPTL3 and proteinuria has not been well clarified.

It is known that podocytes are highly specialized epithelial cells with a complex cellular organization consisting of a cell body, major processes, and foot processes (FPs). Podocyte FPs form a characteristic interdigitating pattern with FPs of neighboring podocytes, creating filtration slits that are bridged by the glomerular slit diaphragm (SD). Proteinuria kidney diseases are typically associated with FP effacement. FP effacement is considered to be a motile event, a characteristic that explains the spread of podocyte FPs on the GBM [9].

Nephrin, which belongs to the Ig super family, plays a key role in the SD and binds adjacent podocyte FPs. In addition to its

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structural role, nephrin acts as a signaling receptor molecule. Thus, it could influence podocyte motility by interacting through cytoskeleton protein signal molecules [10,11].

In our previous studies, we observed that ANGPTL3 was up-regulated in nephrotic kidney tissues using a gene chip technique [12,13]. Through laser micro-cutting techniques, we further detected that ANGPTL3 was only expressed in the kidney glomerulus and not in the kidney tubules [12]. In addition, by using immunohistochemistry, we found that ANGPTL3 was concentrated in the glomerular podocytes of humans and rats [13]. Furthermore, the altered expression of ANGPTL3 in the glomerulus was associated with proteinuria and FP effacement in kidney diseases [14]. These results suggested that ANGPTL3 could be involved in proteinuria development and in podocyte injury.

To better understand ANGPTL3's function in podocytes, we investigated ANGPTL3's effect on podocyte motility as well as the role of nephrin in podocyte motility changes regulated by ANGPTL3. In this study, we found that ANGPTL3 over-expression may contribute to the motility of the podocytes. No effect was observed in those podocytes in which there had been knockdown of ANGPTL3. In addition, adriamycin (ADR) treatment failed to promote the podocyte-directed motility of those cells in which the knockdown of ANGPTL3 had occurred. Our data suggested that nephrin was involved in the signaling mechanism for ANGPTL3-mediated motility in the podocytes.

2. Materials and methods

2.1. Antibodies and reagents

The antibodies and reagents used in this study are listed with their sources in parentheses as follows: monoclonal antibody to glyceraldehydes-phosphate dehydrogenase (GAPDH); rabbit polyclonal antibody to nephrin (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal antibody to ANGPTL3 (R&D Systems, Minneapolis, USA); adriamycin (Pfizer Inc., USA); fluorescein-isothiocyanate-labeled bovine serum albumin (FITC-BSA, Sigma–Aldrich, St. Louis, USA).

2.2. Podocyte culture and treatment

The cultured, immortal mouse podocytes originated in the laboratory of Prof. Mundel P. in the USA. The culture management was performed according to the standards outlined in Prof. Mundel's review [15]. Briefly, the growing immortalized podocytes, which were small and polygonal, were cultured for 5 days at 33 °C, 5% CO_2 in permissive conditions with INF-γ and then for 14 days at 37 °C, 5% CO_2 in restrictive conditions without INF-γ. By the 14th day, the podocyte morphology had changed into spindled cells with small branches. The stable expression of nephrin produced by the podocytes was confirmed by Western blots.

The cells were 70–80% confluent prior to ADR treatment. When podocytes were well differentiated 14 days later, a 2 μl ADR stock solution was added into each well to a final concentration of 0.5 μmol/l. The cells were then cultured for another 24 h and harvested for the next assays.

2.3. Plasmids construction

The pcDNA3.1-ANGPTL3-cDNA plasmid was designated by the Yueda Biotech Laboratory. We purchased the pcDNA™6.2-GW/EmGFPmiR from Invitrogen (Carlsbad, CA). It was produced by the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits and fully complements the mouse ANGPTL3 site and cleaves its mRNA [16]. We used the oligo sequence 5′-TGC TGT ATA GAT GTT CCC TCC AGG AAG TTT TGG CCA CTG ACT GAC TTC CTG GAG AAC ATC TATA-3′, 5′-CCT GTA TAG ATG TTC TCC AGG AAG TCA GTC AGT GGC CAA AAC TTC CTG GAG GGA ACA TCT ATAC-3′.

2.4. Gene transient transfection

In this study, the differentiated podocytes were trypsinized and plated in six-well plates 24 h prior to transfection in RPMI-1640 containing 10% fetal bovine serum. Four micrograms of DNA was transfected with 10 μl of a Lipofectamine 2000 reagent.

2.5. Western blots

The cells were washed with PBS (0.1 M Tris–HCl, pH 7.4; 0.15 M NaCl) and lysed in a buffer containing 50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, phosphate inhibitors (100 mM Na_3VO_4, 10 mM NaF) and protease inhibitors (1 mM PMSF), and the samples were quantified using the Lowery protein assay. For the total proteins, the cells were lysed in 1× SDS lysis buffer (50 mM Tris–HCl, pH 6.8; 2% SDS; 10% glycerol; 1 mM PMSF; and 1 mM Na_3VO_4). An equal amount of lysates (50 μg) were loaded on 10% SDS–PAGE and blotted onto PVDF membranes (Millipore Corp.). The samples were blocked in PBS-Tween (PBST) (0.1 M Tris–HCl, pH 7.4; 0.15 M NaCl; 0.1% Tween-20) with 5% non-fat dry milk, and the membranes were incubated with primary antibodies at appropriate dilutions in PBST with 5% milk overnight at 4 °C. Subsequently, the membranes were washed three times with the PBST solution, followed by incubation with horseradish peroxidase-linked secondary antibody (1:3000) in PBST with 5% milk. The results were visualized by fluorography using an enhanced chemiluminescence system (Perfect Biotech, Shanghai, China).

2.6. Quantitative real-time PCR assays

Quantitative real-time PCR was conducted as described by Vemuganti et al. [17]. The PCR analysis was conducted as described in the instruction manual for real-time PCR Master Mix (TOYOBO Co., Ltd., Osaka, Japan). For each transcript, quantitative real-time PCR was conducted three times in duplication using each of the cDNA samples. The amplified transcripts were quantized with the comparative CT method using β-actin as the internal control. The primers were designed using the Primer Express software (Primer Premier 5.0) based on the GenBank accession numbers, and the sequences were listed: β-actin, 5′-CCT CTA TGC CAA CAC AGT GC-3′, 5′-ATA CTC CTG CTT GCT GAT CC-3′; ANGPTL3, 5′-ACA TGT GGC TGA GAT TGC TGG-3′, 5′-CCT TTG CTC TGT GAT TCC ATG TAG-3′; nephrin, 5′-GCT GGA CGT GCA TTA TGC T-3′, 5′-CTC CTC GTC TTC CCC CAG T-3′.

2.7. Transwell migration assay

Transwell cell culture inserts (pore size 8 μm; Costar Corporation, Corning, NY) were coated with a type-I collagen, rinsed once with PBS and placed in an RPMI 1640 medium in the lower compartment. For each experiment, 1×10^4 of the differentiated podocytes were seeded in the inserts and allowed to migrate for 18 h while being incubated at 37 °C. Non-migratory cells were removed from the upper surface of the membrane, and migrated cells were fixed with cold methanol and stained with Crystal Viole Solution (Sigma–Aldrich). The number of migrated cells was counted using phase contrast microscopy with a 10× objective on an ECLIPSE TS 100 microscope (Nikon, Tokyo, Japan) in the center of a membrane (one field). Data represent the means ± SEM of six independent experiments.