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Vascular endothelial growth factor increases GEnC permeability by affecting the distributions of occludin, ZO-1 and tight juction assembly

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Abstract. – OBJECTIVE: To explore the molecular mechanism of the increased permeability of glomerular endothelial cells (GEnC) stimulated by vascular endothelial growth factor (VEGF).

METHODS: We investigated the permeability-increasing effect and tight junction formation of VEGF by measuring FITC labeled BSA across GEnC monolayer. Then, immunofluorescence and western blot were employed to detect the distributions of occludin and ZO-1.

RESULTS: We found that VEGF increased FITC-BSA permeability. VEGF also caused a loss of occludin and ZO-1 from the endothelial cell junctions, and changed the staining pattern of the cell boundary. Western blot analysis of GEnC lysates revealed that occludin and ZO-1 were redistributed under VEGF treatment.

CONCLUSIONS: These results suggested that VEGF could increase GEnC monolayer permeability by changing distributions and organizations of occludin and ZO-1, which lead to tight junction disassembly. Occludin and ZO-1 appeared to be downstream effectors of the VEGF signaling pathway.

Key Words:
Vascular endothelial growth factor, Glomerular endothelial cells, ZO-1, Occludin, Diabetic kidney disease.

Introduction

Diabetic kidney disease (DKD) is one of the most common and severe microvascular complication in diabetes, which accounts for the end stage renal disease (ESRD) in most developed countries[1]. The development of kidney disease is considered as a multi-step process that resulted from the accumulation of genetic alteration. However, the molecular basis underlying kidney disease and its pathogenesis remain obscure[2,3].

Chronic kidney disease (CKD) is now recognized as a common condition that elevates the risk of cardiovascular disease and kidney failure, as well as other complications[4]. CKD may arise due to a multitude of different factors to renal function, such as haemodynamic changes, cytokines, inflammation and genetic background[5-7]. In kidney diseases, cytokines can be released by circulating leukocytes or from injured kidney cells, which in turn attract and activate leukocytes to specific sites of injury. Therefore, cytokines may act in a systematic, paracrine, or an autocrine fashion[8].

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), plays a central role in angiogenesis. A number of studies have focused on its role in kidney, but its physiological effect is still unknown[9]. Previous studies showed that VEGF also played an important role in angiogenesis/vasculogenesis of glomerulus[10]. VEGF is required to maintain the glomerular filtration barrier structure, and hence for normal renal function of kidney[11,12]. In glomerulus, VEGF is strongly expressed in the cytoplasm of sertoli cells, and some in endothelial cells and intercapillary cells with *in vitro* Test and *in vivo* animal disease model[13]. However, the expression of VEGF receptor is found to mainly occur on glomerular endothelial cells (GEnC) surface using whole binding arrays[14,15]. Microvascular structure of the kidney suggests that VEGF expression in sertoli cells may transfer by heparin sulfate proteoglycan, such as glypican, which take effect through the base membrane to GEnC[16].

The underlying mechanism of VEGF in the pathogenesis of DKD is still unclear. Studies have shown that VEGF is significantly up-regu-

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lated in diabetic renal tissues, but other studies advocated that it is the increased VEGF that caused the occurrence of kidney disease. Thus, the relationship between DKD and VEGF is far from been fully understand[17,18]. Recently studies manifested that in the early stage DKD simulated via the VEGF-overexpressed transgenic mice, even without significantly elevated blood sugar, the increase of VEGF would induce the hypertrophy of glomerular, mesangial proliferative, and glomerular basement membrane thickening[19], followed by the disappearance of split diaphragm and the increase of proteinuria in combination of the ecclasis of sertoli cell[20].

Previously reports have proved that VEGF contributed to the high physiological permeability of glomeruli by VEGF perfusion on isolated glomerular, however, the cellular and molecular mechanism underlying it is still unclear[21]. In our experiment, we found that VEGF could redistribute the tight junction protein, such as ZO-1, Occludin, from cell membrane to intracellular, leading to the destruction of tight juction of endothelial cell and increase of the permeability intercellular.

Materials and Methods

Materials

Recombinant rat VEGF, RPMI 1640, phosphate-buffered saline (PBS, Fisher Scientific, Rodano, MI, Italy), bovine serum albumin (Gibco, Carlsbad, CA, USA), primary antibody: anti-Occlidin polycolonal antibody (Invitrogen, Carlsbad, CA, USA), second antibody: goat anti-rabbit IgG and goat anti-mouse IgG (Invitrogen), fluorescein isothiocyanate-Bull Serum albumin (FITC-BSA) (Sigma, Aldrich, St Louis, MO, USA). Trypsin-EDTA solution, antibiotic antimycotic solution, and all the chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

GEnC Primary Culture

All human studies have been reviewed by Chinese Ethics Committee and have therefore been performed in accordance with the ethical standards of Declaration of Helsinki 2000 as well as the Declaration of Istanbul 2008. The cell line GEnC was a generous gift from Professor Striker in National institutes of Health (NIH, Bethesda, MD, USA). The endothelial cells were maintained in RPMI-1640, supplemented with L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 m/ml streptomycin at 37°C in a humidified incubator containing 5% CO_2. The cell lines used for all experiments were cultured less than 10 generations *in vitro*.

Permeability Detection of GEnC

The endothelial cells were trypsinized with trypsin-EDTA solution to adjust the concentration to be 3.3×10^5 cells/ml. Next, 1.5 ml of the cell suspension was added to each protein-modified substrate, which was placed in a 6-well issue culture plate, with each well filled with 2.5 ml cell culture medium. The plate was, then, incubated for 2 days at 37°C in a humidified incubator containing 5% CO_2, and the substances can be exchanged between the layers of endothelial cells. VEGF (0, 5, 50 ng/ml) were added to culture medium after GEnC grown on Transwell filter (Costar, Bodenstein, Germany) and formed one fusion layer. The 0 ng/ml VEGF was used as a control. Finally, the permeability of the FITC-BSA was detected 30 min following the stimulation of VEGF.

Capture ELISA

Capture ELISA was employed to detect the concentrations of FITC-BSA inner and outer the wells in the plate. Microplate (Nunc) was enveloped with 5 mg/L streptavidin, and then was incubated overnight at 4°C. The well was blocked for 2h with blocking solution, followed by the incubation with HPR streptavidin at room temperature for 1h. Sample was used at a 1:100 dilution in phosphate-buffered saline. Cells were washed with PBS-Tween and stained with tetramethylbenzidine (TMB) at room temperature for 20 min. The reaction was ended with H_2SO_4 (1.8 mol/l) and the absorbance was recorded using a microplate reader at 450 nm.

Immunofluorescence

Cells were seeded on coverslips in six-well plates. After 24h, the cells were washed with PBS twice, permeabilized in 0.1% Triton X-100, and fixed with 4% paraformaldehyde in PBS for 20 min. The paraformaldehyde was quenched with three washes in 100 mM glycine in PBS. Coverslips were permeabilizated and then blocked for 1h in blocking buffer (PBS containing 10% newborn calf serum, 1% bovine serum albumin and 0.02% Triton X-100). After that, the coverslips were incubated with antibodies in staining buffer (blocking buffer lacking bovine

serum albumin) overnight at 4°C. After being washed for three times with staining buffer and then incubated with Alexa 488-conjugated and/or Alexa 546-conjugated secondary antibodies (Invitrogen) for 1h at room temperature, coverslips were then washed twice with 10% FBS/0.02% Triton X-100 in PBS and stained with Hoechst 33 258. Finally, coverslips were washed three times with PBS and one time with water before being mounted on slides. Immunofluorescence was observed with a Leica confocal microscope (Leica, Solms, Germany).

Western Blot

Western blot was performed as described previously[22]. Protein detection was performed with primary antibodies against Occludin, ZO-1, VE-cadherin and JAM-A. Blots were simultaneously incubated with differentially labeled species specific secondary antibodies after being transferred to membranes.

Statistical Analysis

The present data for cell binding density were presented as mean ± SD (standard deviation) from the triplicate experiments, and we determined statistical significance with the Student's *t*-test (one-tailed) at a confidence level of $p < 0.05$ using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

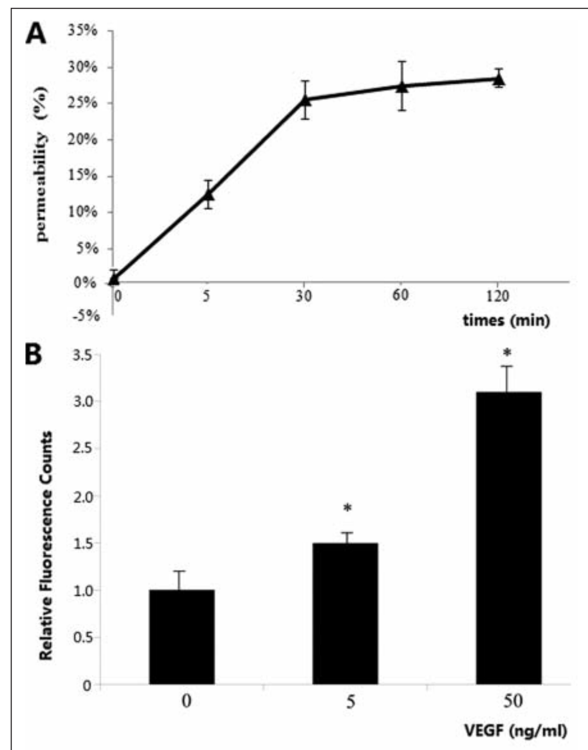

Figure 1. Effect of vascular endothelial growth factor (VEGF) on permeability of glomerular endothelial cells (GEnC). ***A,*** Permeability of GEnC treated with VEGF at different time points. ***B,*** Relative fluorescence counts under different VEGF concentrations. VEGF (50 ng/ml) can increase GEnC permeability by stimulating the cells for 30 min with FITC-BSA leakage significantly increased ($p < 0.05$).

Results

Permeability of GEnC Treated by VEGF

A total of 5×10^5 cells were inoculated in each well, Coomassie Brilliant Blue demonstrated that after 4 days' culture, GEnC formed a tight connection monolayer. Permeability determination showed that albumin could diffuse freely through membranes before cells being inoculated. FITC-BSA appeared a 25% permeability of membrane compared with initial after 30 min of stimulation, and no obvious difference of membrane permeability was found 4 days later. Experiments also showed that continuous extension of growth time could not significantly reduce membrane permeability to albumin (Figure 1A). FITC-BSA leaking experimental results showed that GEnc permeability increased in a concentration-dependent manner, indicating that VEGF can obviously improve the glomerular permeability, and VEGF (50 ng/m[1]) stimulus for 30 min could increase GEnC permeability with a significantly increased FITC-BSA leakage ($p < 0.05$). So VEGF (50 ng/ml) was adopted as a stimulus in the subsequent experiments (Figure 1B).

Distributions of ZO-1 and Occludin in GEnC treated by VEGF

Confocal fluorescence microscopy showed that VEGF (50 ng/ml) could result in the damage of tight junction, redistribution of ZO-1, Occludin, VE-cadherin, and JAM-A in GEnC in 30 min stimulus. As shown in Figure 2, the tight junction of GEnC was damaged (see Figure 2). These data indicated that VEGF may be involved in the redistribution of ZO-1, Occludin, VE-cadherin, and JAM-A.

Western Blot

In order to explore the effect of VEGF (50 ng/ml) on glomerular endothelial cells, we detected the damage of tight junction and the redistribution of ZO-1 and Occludin in cells with

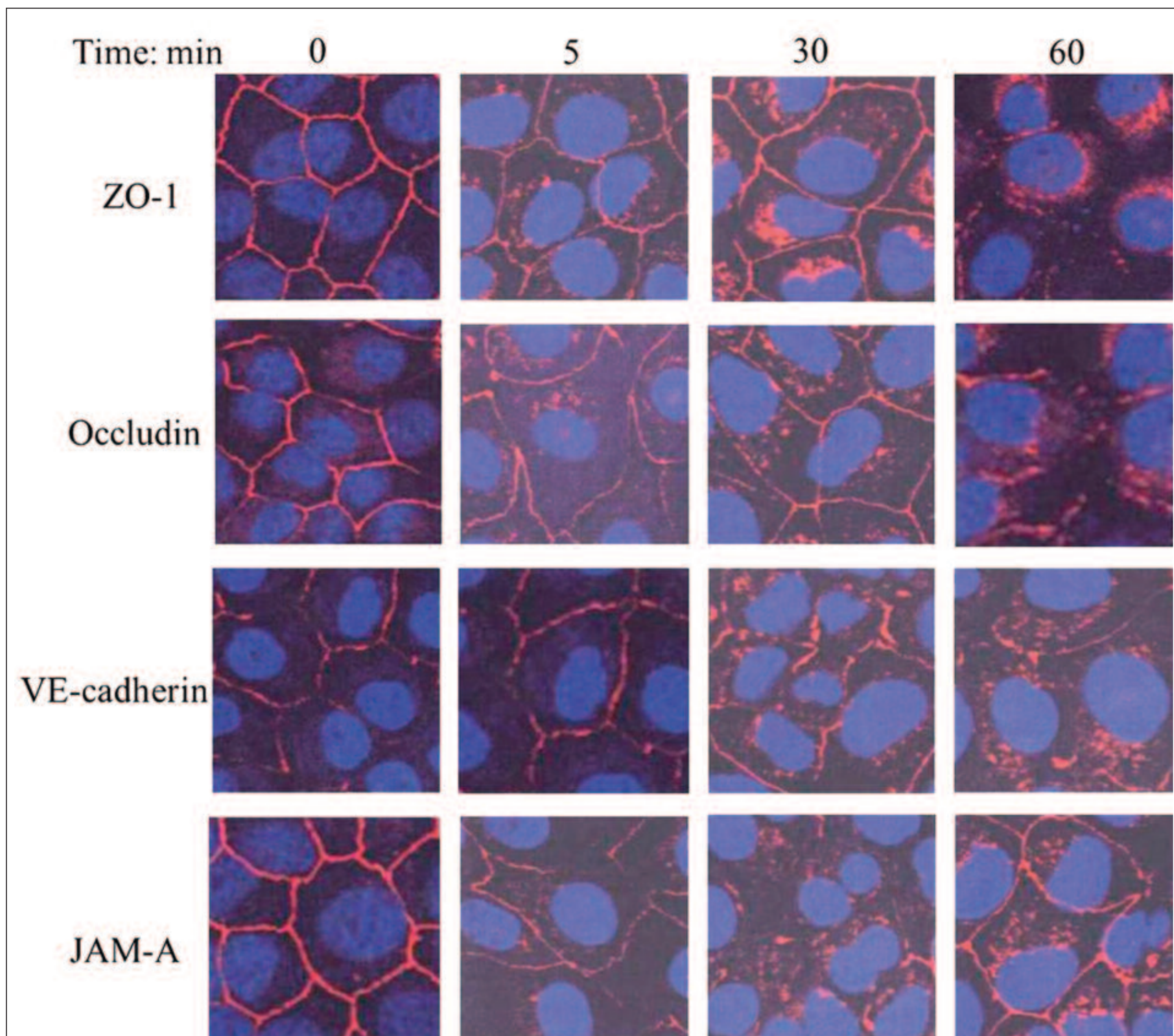

Figure 2. Effect of VEGF on the distribution of occludin, ZO-1, VE-cadherin, and JAM-A and damage of tight junction in GEnC cells (400 × magnification).

western blot at 0 min, 5 min, 30 min, and 60 min. As shown in Figure 3, ZO-1, Occludin, VE-cadherin, and JAM-A decreased in membrane and increased in cytoplasm (see Figure 3). The results indicated that ZO-1 and Occludin accumulated in cytoplasm and reduced in membrane in a time dependent way.

Discussion

VEGF is the most potent vascular permeability factor which regulates the vascular permeability of endothelial cells[23]. It is hypothesized that VEGF controlled glomerular permeability in the normal renal of adult and induces proteinuria in pathological conditions[24,25]. A retrospective survey of patients with primary glomerular disease showed that patients with DKD have increased to be 40% of those with diabetes and is now the leading cause of CKD and end-stage renal disease in the United States[26]. Although a large number of studies have been designed to examine the relationship between VEGF and the DKD[27], the role of VEGF in renal pathophysiology is still poorly understood.

VEGF is mainly distributed in Sertoli cell and epithelial cell[28]. Given the glomerular own special capillary plexus structure and the kidney function, VEGF expressed by Sertoli cell in kidney can across through basilar membrane, sug-

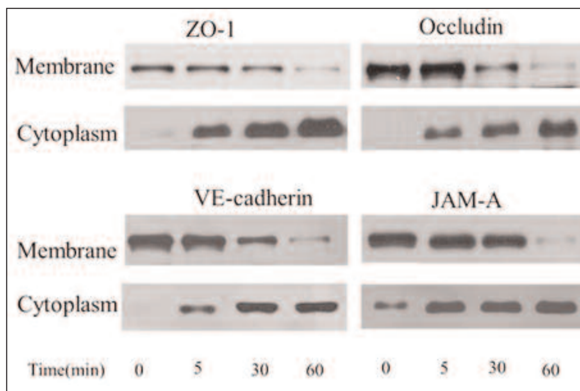

Figure 3. Western blotting showed that the redistribution of ZO-l, Occludin, VE-cadherin, and JAM-A in GEnC cells after stimulation with VEGF.

gesting that VEGF can acts on endothelial cell in a paracrine pattern[29]. Positive correlations between urinary VEGF levels and proteinuria may relate to urinary podocyte loss rather than to a causative link between renal up-regulation of VEGF[27]. Previously studies have shown that VEGF could increase BMEC monolayer permeability by effecting occludin expression and tight juction assembly[30]. VEGF and its receptors are up-regulated in experimental animals and human with type 1 and type 2 diabetes. Inhibition of VEGF have shown beneficial effects on diabetes-induced functional and structural alterations, suggesting a deleterious role of VEGF in the pathophysiology of diabetic nephropathy[27]. In this study, we investigated how VEGF takes an influence on GEnC.

The mechanism by which VEGF disrupts tight jutions and increases permeability is not well understood. Several studies suggested that the binding of VEGF to endothelial cell receptors could induce receptor dimerization, which then stimulates the autophosphorylation of receptors and the phosphorylation of downstream signal transduction proteins[31]. The activation of VEGF on endothelial cell receptors are known to phosphorylate several cytoplasmic proteins, including the ones that contain receptor phosphotyrosine-binding Src homology 2 (SH2) domains which maybe involved in signal transduction[32]. These signals may affect the expression and/or modification of proteins necessary for the maintenance of tight junctions. VEGF also induces rapid and transient elevation in cytosolic calcium in several types of cultured endothelial cells[33]. Elevation of calcium levels may activate calmodulin dependent protein kinases to alter protein phosphorylation and/or affect the actin cytoskeleton, which is important in organizing adhesion junctions and tight junctions.

Tight junctions are highly dynamic structures with permeability, assembly, and disassembly characteristics that can be altered by a variety of cellular and metabolic regulators. Studies using protein kinase activators and inhibitors have revealed that protein phosphorylation plays an important role in tight junction assembly, maintenance, and function in epithelial and endothelial cells[33-35]. Only recently have the phosphorylation levels of the tight junction proteins ZO-1 and occludin been connected with tight junction assembly[36,37], while the role of protein phosphorylation in the structure of GEnC tight junctions is still under investigation. Preliminary results revealed several changes in protein phosphorylation after VEGF treatment but identification of specific occludin and ZO-1 bands had not been made. What's more, we found no influence of VEGF on the phosphorylation of VE-cadherin in this study, even though VEGF could affect the distribution of VE-cadherin.

The VEGF-induced permeability increase could also be caused by an increase of endocytotic activity or in the transcellular trafficking in GEnC. The degree of the contribution of the paracellular and transcellular pathways to the permeability increase described here remains to be determined.

Conclusions

Although the mechanism or signal transduction pathway for the permeability-increasing effect of VEGF is not well understood, it is certain that VEGF increases the permeability of the GEnC monolayers and modifies the localization of the tight junction proteins occludin and ZO-1. We expect that an understanding of the mechanisms of VEGF induced permeability will enable the use of VEGF and related agents to facilitate drug delivery to kidney, as well as the development of therapeutic drugs to intervene in the signal transduction pathway of VEGF for the treatment of kidney and related diseases.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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