Original Article

The Effect of siRNA-VEGF on the Growth of REC in Retinal Pigment Epithelial Cell and Retinal Endothelial Cell Co-culture System

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Abstract

Purpose: To investigate the effect of small interfering RNA (siRNA)targeting VEGF of retinal pigment epithelium (RPE) cells on the growth activity of human retinal vascular endothelial cells (RECs) under a co-culture system.

Methods: By applying the vector (pGPU6)-based siRNA plasmid gene silence system, we specifically silenced VEGF expression of RPE cells (ARPE-19) through plasmid (pGPU6-VEGFA-siRNA)transfection. Reverse transcription polymerase chain reaction(RT-PCR) was applied for selecting the most efficient siRNA segment among three pGPU6-VEGF-siRNA groups (siRNA-1, siRNA-2 and siRNA-3). Treated RPE cells were co-cultured with RECs in a co-culture system made up of a 24-well culture plate and transwell inserts assembled inside During 7-day culture period, the growth capacity of RECs were observed and tested in the form of cell counting assay. Three groups were established in this study: RPE cells transfected with pGPU6-VEGF-siRNA and co-cultured with RECs (group A), RPE cells transfected with siRNA null vector and co-cultured with RECs (group B), and RECs cultured alone (group C).

Results: After transfection, VEGF expression levels of RPE cells in three pGPU6-VEGF-siRNA groups (siRNA-1, siRNA-2 and siRNA-3)evaluated by RT-PCR were 2.56±0.45, 1.17±0.38 and 4.39±0.51, respectively (n=10). siRNA-2 was selected as the foremost segment for transfection (P< 0.05, SNK-q test). During the 7-day co-culture period, the influence upon the growth of RECs was observed. Growth curve of RECs under co-culture showed a lower growth rate in group A than in group B (P<0.05, dunnett's test), but no significant difference between group A and group C was noted (P>0.05, dunnett′s test). RECs in group A proliferated much faster during the first four days post-transfection.

Conclusion: Delivery of siRNA targeting VEGF plays an efficient role in down-regulating VEGF expression in RPE cells, therefore modulating the growth activity of RECs under a co-culture system in vitro. The application of this technique may provide novel evidence for the prevention and treatment of retinal neovascularization diseases. (***Eye Science 2011;26:75-79***)

Keywords: VEGF; siRNA; RPE cell; co-culture; retinal endothelial cell

Introduction

Retinal neovascularization contributes to various types of vitreo-retinal diseases, such as proliferative diabetic retinopathy and retinal vein occlusion[1-4]. Its occurrence and progression have a close relationship with the dysfunction of blood-retinal barrier (BRB), where retinal pigment epithelium (RPE) cells and retinal vascular endothelial cells (RECs) have been considered as particular components[3,5,6]. As the essential cause of retinal neovascularization, vascular endothelial growth factor (VEGF) have been discovered to notably affect the growth activity and function implementation of RECs and RPE cells respectively in vitro[7,8]. Moreover, RECs and RPE cells in vivo are not isolated, which also maintain a close mutual correlation through VEGF and other cytokines and play a crucial role in keeping retina inner-environment homeostasis[9-11]. So, in the present study, we aimed to simulate a physical and biological condition in vitro providing better illustrations for mutual cell interaction between RECs and RPE cells. The co-culture system constructed in the study was composed of two parts: a 24-well culture plate and built-in transwell inserts. The transwell inserts divided each well into upper and lower chambers,

DOI: 10.3969/g.issn.1000-4432.2011.02.004
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thus RECs and RPE cells could be cultured separately in the same well but contacted with each other through some macromolecular materials released in the medium. Together with applying VEGF-siRNA technique to silence the expression of VEGF gene in RPE cells, we aimed at finding more evidence about the modulation mechanism of VEGF signaling between RECs and RPE cells, and making more contributions towards the prevention and treatment of retinal neovascularization diseases.

Materials and methods

Culture of human REC and RPE cell

Human RECs and RPE cells (ARPE-19) were obtained from Sciencell Research Laboratories (San Diego, CA, USA) and American Type Culture Collection (ATCC, Manassas, VA, USA), respectively. Each type of cells were seeded at a density of 5×10^5/ml, and separately maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA), supplemented with 20% heat-inactivated fetal calf serum (FBS, Invitrogen-Gibco, San Diego, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured at 37°C under a humidified atmosphere containing 5% CO_2. After subconfluent to a single layer, cells were detached with trypsin-EDTA (Gibco, USA) for 5 min and subcultured in the same condition. Cells obtained at exponential phase were used for the experiment.

Recombinant pGPU6-VEGFA-siRNA construction

Small-interfering RNA (siRNA) against VEGF (GeneBank, NM_003376) was constructed based on the principle of short hairpin RNA (shRNA) construction and synthesized by Sangon Company (Shanghai, China) using pGPU6/GFP/Neo siRNA Expression Vector (Genepharma Corporation, NY, USA). Three sequences were designed as the target interfering segments and described as follows: siRNA-1: pGPU6/GFP/Neo-VEGFA-1425 (bases 1425-1484 of NM003376): (forward 5'-CAC CGC CAA AGA AAG ATA GAG CAA TTC AAG AGA TTG CTC TAT CTT TCT TTG GTT TTT TG-3', reverse 5'-GAT CCA AAA AAC CAA AGA AAG ATA GAG CAA TCT CTT GAA TTG CTC TAT CTT TCT TTG GC-3'); siRNA-2: pGPU6/GFP/Neo-VEGFA-775 (bases 775-833 of NM003376): (forward 5'-CAC CGG GAG GAG GAA GAA GAG AAT TCA AGA GAT TCT CTT CTT CCT CCT CCC TTT TTT G-3', reverse 5'-GAT CCA AAA AAG GGA GGA GGA AGA AGA GAA TCT CTT GAA TTC TCT TCT TCC TCC TCC C-3'); siRNA-3: pGPU6/GFP/Neo-VEGFA-1378 (bases 1378-1437 of NM003376): (forward 5'-CAC CGA CAT AGG AGA GAT GAG CTT TTC AAG AGA AAG CTC ATC TCT CCT ATG TTT TTT TG-3', reverse 5'-GAT CCA AAA AAA CAT AGG AGA GAT GAG CTT TCT CTT GAA AAG CTC ATC TCT CCT ATG TC-3').

After methylation modification to the VEGF siRNA terminal sequences, the final recombinant pGPU6-VEGF-siRNA were acquired. Inserted sequences were confirmed by DNA sequencing to be identical to the construction. Sequences selected were screened via BLAST search and did not show any homology to other human genes. A negative (nonsilencing) control psiRNA was also purchased from Sangon Company (Shanghai, China).

pGPU6-VEGF-siRNA transfection of RPE cell

Transfection was performed at approximately 80% confluency of RPE cells in six-well plates (Corning, NY, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In brief, a total of 2×10^5 RPE cells were seeded into each well in DMEM containing 10% calf serum without antibiotics the day before transfection. A 2.5:1 ratio of lipofectamine-psiRNA-VEGF complexes were prepared and added to the RPE cells.

RT-PCR analysis

After incubation for 48 hours, total RNA of each transfection group (n=10) were isolated to determine the superior segment for VEGF interfering. Total RNA was extracted using the RNeasy total RNA Kit (Qiagen, Hilden, Germany), and 1 μg total RNA was used for the amplification of VEGF mRNA. The primer sets used were as follows: forward 5'-AAT CGA GAC CCT GGT GGA CA-3', reverse 5'-TTA ACT CAA GCT GCC TCG CC-3'. Relative quantities of mRNA were normalized against glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, primer

sequences: forward 5′-CGT GGA AGG ACT CAT GAC CA-3′, reverse 5′-TCC AGG GGT CTT ACT CCT TG-3′). Reactions were performed in triplicate in 50 μl volume, according to the Promega "Access RT-PCR Introductory System" kit protocol (Southampton, UK). Briefly, RT reactions were incubated at 45℃ for 45 min. PCR Reactions were amplified and quantified in an Applied Biosystems Geneamp PCR 9700 Thermocycler (Hayward, CA, USA) in a 96-well plate at 94° C for 2 min, followed by 40 amplification cycles, comprising of 30 s at 94°C, 1 min at 52.5°C and 2 min at 68°C, and a final extension at 68℃ for 7 min. All RT-PCR products, including no-template and RT-minus controls, were run in triplicate, using a ChemiDoc system and Lab-Works software (UVP, Upland, CA).

Co-culture of transfected human RPE cells and RECs

Transfected human RPE cells were collected by the treatment of trypsin-EDTA digestion for 3 minutes, and re-suspended in DMEM at a density of 2×10^4 cells/ml. A total of 600 μl of suspensions were added to the lower chamber of a 24-well plate with transwells (3.0 μm pore size; Corning Costar Corp., Cam bridge, MA, USA). RECs obtained in the same way were re-suspended in DMEM at a density of 1×10^5 cells/ml, and 100μl were added to the inserts (upper chamber) of the 24-well plate. During the one week co-culture period, we observed changes in cell morphology, collected cells in the inserts each day, counted the number and drew the growth curve to indicate the growth capacity of REC. Meanwhile, Data presented were from means of triplicates from three wells of each group. Four groups established in this study were as follows: RPE cells transfected with VEGF-siRNA and co-cultured with RECs (group A), RPE cells transfected with empty plasmid and co-cultured with RECs (group B), and RECs cultured alone (group C).

Results

Screening plasmid for transfection

The expression levels of siRNA-1, siRNA-2 and siRNA-3 were 2.56 ±0.45, 1.17 ±0.38, and 4.39 ± 0.51, respectively (n =10). Significant differences were documented among control group and each of the three experimental groups (SNK-q test, $P<0.05$). Therefore, siRNA-2 was chosen for the subsequent experiment due to higher transfection efficiency (Figure 1).

RECs growth under co-culture system with transfected RPE cells

The RECs cultured alone (group C) were spindle-shaped and exhibited cobblestone-like arrangement after confluence (Figure 2A). During the seven days' culture, RECs in group A displayed a significantly decreased growth rate than that in group B ($P<0.05$, dunnett's test), but no significant difference on growth curve was noted compared with group C ($P>0.05$, dunnett's test). The results indicated that RECs in group A grew slower than those in group B, but had similar growth rate with those in group C, which exhibited few morphological changes (Table 1, Figure 2B).

Discussion

In a multicellular organism, cell signaling plays an important role in determining the relationship among contiguous cells. Cytokines, always polypeptides or proteins, can be produced through paracrine or autocrine to perform signal transmission, and directly affect cell proliferation, differentiation or other functions. As the main composition of BRB, RPE cells intimately correlate with their adjacent RECs, and their relationship is especially vital in the pathophysiology of various types of retinal neovascularization diseases[10]. VEGF, which mainly acts on vascular endothelial cells, functions as an essential cytokine for angiogenesis and also regulates the growth capacity of RPE cells and RECs[12]. So, inhibiting the secretion of VEGF in vivo will probably provide novel ocular treatments and perspectives for evaluation of studies of retinal neovascularization diseases[13].

Previous studies have demonstrated that VEGF is able to directly promote the growth capacity and proliferation of RPE cells and RECs, separately[1,14,15]. Simultaneously, blocking VEGF expression suppresses the cell growth capacity notably. However, RPE cells and RECs are an organic unity as the main components of BRB in vivo, and never exist separately. Therefore, in the present study, we con-

structed a co-culture system to simulate the physical and biological condition of cell interaction in vitro, in order to better illustrate cell interactions. In this system, RPE cells and RECs shared a mutual medium but without direct connection. By simulating this physical living state, we tried to illustrate the changes of growth capacity on RECs when cultured with RPE cells, and how these two cell lines responded when VEGF expression of RPE cells was silenced. Thus the co-culture system might help us to clarify the mechanisms of VEGF cells signaling and place special emphasis on illuminating the underlying target molecules and relevant intercellular signalling pathways.

In our present study, we constructed the co-culture system to observe the influence of RECs under RPE cells targeting or disruption of VEGF signalling. The system was mainly composed of a 24-well culture plate and several transwell inserts assembled in the plate. The transwell inserts divided each chamber into upper and lower parts, and each part was used to culture RECs or RPE cells individually. At the bottom of the inserts there was a polycarbonate membrane with filter pore size of 3 μm and large molecules like cytokines and other nutritive materials freely passed through except cells. Therefore, this construct provided an appropriate method of culturing two types of cells respectively, but mutual material exchange and signal connection allowed us to better observe independent growth capacity of both types of cells under mutual effects.

Previous studies also showed that VEGF explicitly enhanced the growth and proliferation of RPE cells and RECs when cultured alone, and blocking the expression of VEGF could notably depress the cell growth viability. In our study, we found that under co-culture condition, RPE cells without VEGF-siRNA treatment could greatly promote the growth of RECs, as shown in group B. However, when VEGF function was silenced, RECs co-cultured with RPE cells (group A) exhibited similar growth rate as RECs cultured alone (group C) ($P>0.05$, dunnett's test). The results indicated that along with the interfered expression of VEGF, the influence of RPE cells upon RECs growth capacity was greatly decreased. In addition, comparing REC growth between group A and group C also demonstrated the effectiveness of VEGF-siRNA. It has been suggested that VEGF is one of the essential promotors that causes the development and progression of retinal neovascularization[10]. Under normal circumstances, VEGF concentration was always too low to promote cell proliferation. However, under certain pathologic conditions like hypoxia, VEGF secretion increased significantly, and was able to regulate directly the neovascularization development[4]. As one of the cells that produce VEGF, RPE cell itself bears two kinds of VEGF receptors (VEGF-R1 or FLT-1, and VEGF-R2 or FLK-1/KDR), which make its growth capacity conversely regulated by VEGF[16,17]. Therefore, we chose RPE cells as the target cells for VEGF-siRNA transfection in this study. As one kind of pathologic state, retinal neovascularization stimulates RPE cell proliferation and increases VEGF secretion. Through VEGF receptors, RPE cell proliferation is enhanced/promoted and results in more VEGF production, which in turn causes more VEGF release and form a vicious circle. So blocking VEGF expression and its function to RPE cell can be considered as an advanced and effective method of interrupting disease progress.

In conclusion, co-culture system has been proved to be an advanced method of investigating cell signallings between RPE cells and RECs. By using this system, we concluded that VEGF is one of the factors transmitting signals between RPE cells and RECs and regulating RECs' growth. Delivery of siRNA targeting VEGF is seemingly efficient in down-regulating VEGF expression in RPE cells, consequently inhibiting the growth capacity of RECs, which may lead to improved techniques for the prophylaxis and treatment of retinal neovascularization diseases.

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