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Repulsive guidance molecule A suppresses angiogenesis

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ABSTRACT

The repulsive guidance molecule-a (RGMa) is a membrane-associated glycoprotein that has diverse functions in the developing and adult central nervous system. Here, we show that RGMa suppresses new blood vessel formation. Treatment of human umbilical artery endothelial cells (HUAEC) on Matrigel with recombinant RGMa inhibits vascular endothelial growth factor (VEGF)-induced and VEGF-independent tubular formation and migration. RGMa enhances adhesion presumably through dephosphorylation of focal adhesion kinase (FAK) at tyrosine-397. Neogenin, an RGMa receptor, in HUAEC is required for the effect of RGMa. *In vivo* Matrigel plug assay reveals that treatment with recombinant RGMa suppresses angiogenesis. Thus, we conclude that RGMa inhibits angiogenesis *in vitro* and *in vivo* suggesting that its manipulation would be an efficient therapeutic strategy for pro-angiogenic conditions.

1. Introduction

Angiogenesis is the process of new blood vessel formation from pre-existing vessels. A balance between factors that induce and inhibit vascular formation tightly controls it [1,2]. Anti-angiogenesis therapy is considered a promising approach for pro-angiogenic diseases [3–5], but many questions remain unanswered [3]. Age-related macular degeneration (AMD) is closely associated with pathological angiogenesis characterized by abnormal blood vessel [6–8]. Evidence indicates VEGF is one of the key regulators for angiogenesis in AMD [2,9,10]. Therapeutic interventions that target VEGF signaling have proven to be effective inhibiting pathological angiogenesis [3,11,12]; however, some individuals with AMD become refractory to further anti-VEGF therapy [6]. Thus, identifying alternative pathways that can target novel anti-angiogenic agents is beneficial.

Reports identified netrins regulate angiogenesis. These secreted molecules have been implicated in neural network formation and vessel pathfinding [1,9]. Netrin-4 inhibits angiogenesis through one of its receptors, neogenin [8,13]. Therefore, we focused on RGMa, a ligand of neogenin, in the similar manner as in netrin-4 verifying an efficient therapeutic strategy for pro-angiogenic conditions.

Repulsive guidance molecule-a (RGMa) is a glycosylphosphatidylinositol-anchored membrane protein that plays an important role in axon guidance in the visual system [14]. In vertebrates, RGMa is highly homologous to chick RGM [15,16]. Neogenin serves as a receptor for RGM as well as netrins [17,18]. RGMa binds to neogenin and mediates its repulsive activity toward axons [19], playing roles in both the developing and adult central nervous systems. RGMa regulates cell fate in the developing chick retina [20] and inhibits axon regeneration after spinal cord injury in adult rat [21]. These findings prompted us to hypothesize that RGMa may act as a functional cue in angiogenesis.

2. Materials and methods

2.1. Endothelial cell cultures and siRNA transfection

HUAEC were cultured in Complete Medium Kit with serum and CultureBoost-R (Cell Systems Corporation, Kirkland, WA, USA). The kit consists of 500 mL CSC medium with 10 mL CultureBoost-R (containing human recombinant growth factors). Transient transfection of HUAEC was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 36 h, the 0.5% FBS/CSC medium was changed, and the cells were subjected to western blotting and real-time PCR. siRNA for neogenin has the following sequence: #1, 5′-rCUrGrGUUrAUrCrArGrCrArAUrGrCUrATT-3′; #2, rCrArAUUrCrCrAUrGrGrAUrArGrCrArAUTT-3′. Negative control siRNA (Ambion, TX, USA), which had no sequence similarity to the human gene, was used as a control siRNA.

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2.2. Tube formation assay

Matrigel was thawed on ice overnight. HUAEC (3×10^4) were applied on top of 300 μL Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) in a 24-well plate and incubated at 37 °C 5% CO_2. After 1 h, HUAEC were treated with or without 50 ng/mL VEGF (R&D Systems, Minneapolis, MN, USA), 500 ng/mL RGMa (R&D Systems), and 1000 ng/mL Netrin-4 (R&D Systems) before they were further incubated for 16 h. Tube formation by HUAEC was observed after plating endothelial cells on Matrigel. Images were acquired on a microscope (BZ-9000, Keyence, Tokyo, Japan), and the numbers of tubes were counted. Photographs of 10 representative fields were used for quantification.

2.3. Cell migration assay

Transwell cell culture chamber inserts (polycarbonate, tissue culture-treated, 6.5-mm diameter, 8-μm pore size) were used for migration assays (Corning Costar, Corning, NY, USA). HUAEC were starved in CSC medium containing 0.5% FBS after 24 h. Next, 3×10^4 cells/100 μL were transferred into the upper inserts and cultured in CSC medium containing 0.5% FBS with or without RGMa at 37 °C for 16 h. After fixation with 4% PFA, the transwell filters were incubated for 30 min in a solution of 4,6-diamidinophenylindole (DAPI, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The upper surface filter (non-migrating cells) were removed and counted $DAPI^+$ cells on the lower surface. In a checkerboard analysis, chemoattractants (RGMa and VEGF together) were added to lower and/or upper chambers to assess chemotaxis (directional) and chemokinesis (random) migration.

2.4. Cell adhesion assay

96-well plates were coated with 0.02% PLL overnight and dried. After, blocked with CSC medium containing 1% BSA for 1 h at 37 °C. HUAEC were pretreated with or without RGMa for 30 min. 1×10^4 cells/well in CSC/0.1% BSA (serum starved) were plated in triplet and incubated for 1 h at 37 °C. After washing with PBS, cells were fixed with 3% PFA for 1 h at 4 °C. Then HUAEC were stained with 0.5% crystal violet in PBS (100 μL) at room temperature for 20 min. After washing extensively with PBS, the absorbance was measured (OD at 590 nm) using a microplate reader (SpectraMax M2, Molecular Devices Japan, Tokyo, Japan).

2.5. Cell proliferation assay

HUAEC (5×10^3 cells/well, 100 μL) were seeded into a 96-well plates contained with serum-free CSC medium treated with RGMa at 37 °C 5% CO_2 for 24 h in the incubator. Then, 10 μL cell counting kit-8 (CCK-8) solution (Sigma–Aldrich, St. Louis, MO, USA) was added to each well, being careful not to introduce bubbles. The cells were further incubated for 1 h. After incubation, the absorbance was measured (OD at 450 nm). The relative growth rate was calculated as treatment absorbance/untreated control absorbance.

2.6. Real-time PCR

Total RNA was extracted from HUAEC using an RNeasy kit (Qiagen, Venlo, The Netherlands) and reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). mRNA expression was determined by real-time PCR using a 7300 Fast Real-Time PCR system (Applied Biosystems). SYBR Green assays were used to quantitate neogenin expression. A total sample volume of 20 μL was used for SYBR green assays, which contained a 1 × final concentration of Power SYBR green PCR master mix (Applied Biosystems), 400 nM gene-specific primers, and 2 μL cDNA template. The PCR cycles began with a UNG digestion stage at 50 °C for 2 min, an initial denaturation period at 95 °C lasting for 10 min, followed by 40 cycles at 95 °C for 15 s, and an annealing phase conducted at 60 °C for 1 min. The relative mRNA expression was normalized measuring the amount of human GAPDH mRNA in each sample. The results of cycle threshold values (Ct values) were calculated by the $\Delta\Delta C_T$ method to obtain the fold differences. Primer sequences for real-time PCR were as follows: The primer pairs used for PCR are as follows: GAPDH, Forward: 5′-tgacgtgccgcctggagaaa-3′, Reverse: 5′-gacttcccgtagaacccgatgtga-3′ and neogenin, Forward: 5′-cccatgtctgaagctgtccaa-3′, Reverse: 5′-ctgaggcttgatcattaggcatt-3′.

2.7. Western blotting

To study FAK phosphorylation, HUAEC were stimulated with or without 50 ng/mL VEGF and 2 μg/mL RGMa (R&D Systems) for 30 min. Cells were lysed using a mixture containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM Na_3VO_4, 1 mM NaF, and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). The homogenate was centrifuged at 10,000 × *g* for 10 min. Protein concentration was measured using a bicinchoninic acid protein (BCA) assay kit (Pierce/Thermo Scientific, Rockford, IL, USA). Cell lysates were boiled in sample buffer for 5 min, and proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% bovine serum albumin (BSA) (Sigma–Aldrich) in phosphate buffered saline containing Tween (PBS-T) and incubated for 1 h at room temperature. Then incubated anti-FAK antibody (1:500; Santa Cruz Biotechnology) or anti-FAK (pY397) antibody (1:500; Invitrogen) in PBS-T containing 1% BSA overnight at 4 °C. After washing in PBS-T, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. Signals were detected with an enhanced chemiluminescence system (GE Healthcare, Little Chalfont, UK) and quantified using an LAS-3000 image analyzer (Fuji Film, Tokyo, Japan), according to the manufacturer's specifications. The relative phosphorylation levels of FAK were normalized to the signal intensity of total FAK.

2.8. Subcellular (membrane) fractionation

HUAEC were stimulated with or without 2 μg/mL RGMa for 30 min before harvesting. Fractionations were prepared using the Focus Global Fractionation (G Biosciences, St. Louis, MO, USA) as per manufacturer's instruction. Protein concentration was quantitated using the BCA protein assay kit (Pierce) and separated by SDS-polyacrylamide gel electrophoresis (as above in western blotting). Membranes and other (including nucleus and cytoplasm) were probed with the following primary antibodies: anti-α tubulin antibody (1:200; Santa Cruz Biotechnology), anti-integrin β-1 antibody (1:5000; BD Biosciences), and anti-neogenin antibody (1:200; Santa Cruz Biotechnology) in PBS-T containing 1% BSA. After washing in PBS-T, the membrane was incubated with either horseradish peroxidase (HRP)-labeled secondary antibodies: conjugated anti-rabbit IgG antibody (1:5000; Cell Signaling Technology) or conjugated anti-mouse IgG antibody (1:5000; Cell Signaling Technology).

2.9. Immunocytochemistry

HUAEC were stimulated with 2 μg/mL RGMa for 2 h. Then, the cells were fixed with 4% paraformaldehyde at room temperature for