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Functional interaction between CTGF and FPRL1 regulates VEGF-A-induced angiogenesis

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ABSTRACT

Vascular endothelial growth factor-A (VEGF-A) is a master regulator of angiogenesis that controls several angiogenic processes in endothelial cells. However, the detailed mechanisms of VEGF-A responsible for pleiotropic functions and crosstalk with other signaling pathways have not been fully understood. Here, we found that VEGF-A utilizes the connective tissue growth factor (CTGF)/formyl peptide receptor-like 1 (FPRL1) axis as one of its mediators in angiogenesis. Using a proteomic approach, we found increased secretion of a matricellular protein, CTGF, from VEGF-A-treated human umbilical vein endothelial cells (HUVECs). Then, we studied the effect of CTGF binding to FPRL1 in VEGF-A-induced angiogenesis. CTGF directly binds to FPRL1 through a linker region and activates the downstream signals of FPRL1, such as increase in extracellular signal-regulated kinase (ERK) phosphorylation and intracellular Ca^{2+} concentration. We found that linker region-induced FPRL1 activation promotes the migration and network formation of HUVECs, while disruption of FPRL1 inhibits VEGF-A-induced HUVEC migration and network formation. In addition, similar results were observed by the chorioallantoic membrane (CAM) assay based evaluation of angiogenesis *in vivo*. To summarize, our data reveal a novel working model for VEGF-A-induced angiogenesis via the VEGF-A/CTGF/FPRL1 axis that might prolong and enhance the signals initiated from VEGF-A.

1. Introduction

Vascular endothelial growth factor A (VEGF-A) is a prominent regulator of blood vessel formation. During angiogenic processes, VEGF-A modulates several cellular functions of endothelial cells including survival, proliferation, migration, and tube formation [1,2]. VEGF-A-induced effects are initiated by its binding to VEGF receptors (VEGFRs), a family of receptor tyrosine kinases (RTKs) expressed in the endothelial cells [3,4]. Activated VEGFR transduces the signal into endothelial cells through complex signaling cascades that mediate various cellular functions required for angiogenesis. Multiple biological outcomes of VEGF-A are conferred by many downstream factors and depend on the cellular context [5]. A few reports suggested crosstalk with other signaling pathways that can reinforce and diversify the biological effects of VEGF-A [6,7]. However, the mechanism employed by VEGF-A to exert its pleiotropic functions is still not fully understood.

Formyl peptide receptor-like 1 (FPRL1) is a member of the human formyl peptide receptor (FPR) gene family that can bind to natural and synthetic peptides of various sequences and plays a role in host defense against pathogen infection [8,9]. FPRL1 is promiscuous with respect to agonist selectivity and pleiotropic cellular functions. A number of natural and synthetic ligands, including serum amyloid A, $A\beta_{1-42}$, lipoxin A4, and $Prp_{106-126}$, and various bacterial and synthetic peptides have been shown to interact with FPRL1 and mediate inflammation and innate immunity [8–11]. Although the contribution of FPRL1 to endothelial cell function and angiogenesis has been reported, detailed mechanisms and the relationship with other angiogenic signaling pathways are yet to be elucidated [12,13].

During a search for novel FPRL1 functions in HUVECs, we accidentally found that the blocking of FPRL1 signaling affected the long-term angiogenesis induced by VEGF-A. Therefore, we hypothesized that a secreted factor induced by VEGF-A activates FPRL1-mediated angiogenesis. In this study, using proteomic analysis, we identified CTGF to be a mediator for the functional interaction between VEGF-A and FPRL1. Increased expression and secretion of CTGF by VEGF-A treatment activated FPRL1, which was required for the cellular functions of endothelial cells and for angiogenesis *in vivo*. Our data suggest that the CTGF/

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FPRL1 pathway is involved in the amplification and propagation of VEGF-A-mediated angiogenic signaling and that CTGF is a mediator of the functional interaction between VEGF-A and FPRL1 that is necessary to induce angiogenesis.

2. Materials and methods

2.1. Materials

Phospho-ERK1/2 (Thr^{202}/Tyr^{204}) and ERK1/2 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Human CTGF antibody and anti-Flag antibody were obtained from Abcam (Cambridge, MA) and Sigma (St. Louis, MO), respectively. Recombinant human CTGF was purchased from BioVendor Laboratory Medicine Inc. (Brno, Czech Republic). Recombinant human VEGF-A_{165}, anti-VEGF-A mAb, anti-VEGFR-1 mAb, and anti-VEGFR-2 mAb were obtained from R&D Systems (Minneapolis, MN). siRNAs against FPRL1 and luciferase were synthesized by Dharmacon Research, Inc. (Chicago, IL), and WKYMVm, WRWWWW, and biotinylated WKYMVm were synthesized by A&PEP Inc. (Seoul, Korea), and exceeded 95% purity. The linker peptide (EWVCDEPKDQTVVGPALAAYRLEDTFGPDPTMIRANCLV-NH_2) containing the amino acid sequence of the human CTGF linker region was synthesized by Anygen Co. Ltd. (Gwangjoo, Korea) with 99.1% purity.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from the umbilical cord by collagenase treatment as previously described [35] and cultured in 0.2% gelatin-coated dishes using Medium 199 containing 1% penicillin/streptomycin and 20% (*v*/*v*) heat-inactivated fetal bovine serum. For experiments, cells were grown to sub-confluence and used from passages 4 through 7. FPRL1-expressing rat basophil leukemia (RBL)-2H3 (FPRL1/RBL) cells, FPR-expressing RBL-2H3 (FPR/RBL) cells, and vector-transfected RBL-2H3 (vector/RBL) cells were maintained in high glucose DMEM supplemented with 1% penicillin/streptomycin, 20% (*v*/*v*) heat-inactivated fetal calf serum and G418 (500 μg/ml) [14]. Cells were grown at 37 °C in an atmosphere containing 5% CO_2.

2.3. Conditioned medium

Conditioned medium (CM) was collected from HUVECs in Medium 199 without FBS. The collected medium was centrifuged to remove any residual cells and stored at −80 °C until use.

2.4. Western blot analysis

Vector/RBL cells, FPR/RBL cells, FPRL1/RBL cells and HUVECs were cultured to sub-confluence and serum-starved. The stimulated cells were washed twice in PBS, dissolved in sample buffer (50 mM Tris–HCl, 100 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 50 mM NaF, 1 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin), boiled, separated via SDS-PAGE, and transferred to nitrocellulose membranes. After immunoblot analysis with specific antibodies, the membrane was visualized with a chemiluminescence substrate (Amersham Pharmacia). Quantification was conducted blindly using ImageJ (http://rsb.info.nih.gov/ij).

2.5. Identification of FPRL1 activating protein

HUVECs-CMs were loaded on HLB cartridges (waters) and then separated using a reverse phase (RP) C18 column. An RP C18 HPLC column (218 TP5215; 2.1 mm × 150 mm, Vydac) was equilibrated with water/0.1% TFA. A 0–100% gradient of ACN/0.1% TFA was applied and 150 μl fractions were collected. The active fraction was incubated with trypsin overnight. To obtain the MS and MS/MS data of the active fraction, we used nano-LC MS consisting of an Ultimate HPLC system (LC Packings) and a QSTAR PULSAR I hybrid Q-TOF MS/MS system (Applied Biosytems/PE SCIEX) equipped with a nano-ESI source. The QSTAR was operated at a resolution of 8000–10,000 with a mass maintained for 24 h. The voltage of the spray tip was set at 2300 V. All of the masses detected by QSTAR were calculated using Analyst QS software provided by Applied Biosystems (AB). For MS/MS analysis, the common mass values spectra were analyzed using MASCOT search engine version 1.7 (in-house) against a non-redundant database to obtain sequence information. The accepted MASCOT results had to have a higher MOWSE score than that indicated for a random match ($p < 0.05$).

2.6. Plasmid construction, transfection, and protein purification

E. M. De Robertis (University of California, Los Angeles, CA) provided the full length and deletion mutants of Xenopus CTGF cloned into a $pCS2^+$ expression vector containing a chordin signal peptide until Ala41, followed by the Flag-tag sequence [15]. Flag-tagged secreted proteins were obtained by transient transfection of human 293 T cells using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Full-length and deletion mutants Flag-CTGF from HEK293T cells were affinity purified with anti-Flag M2 affinity gel column and eluted with Flag peptide according to the manufacturer's directions. The purity of the proteins was determined by silver staining with a Silver Stain Plus kit (Bio-Rad).

2.7. Biotinylation and flow cytometry

To assess the binding level of the linker peptide corresponding to the linker region of human CTGF, labeling of the linker peptide was performed using the EZ-Link Micro Sulfo-NHS-LC-Biotinylation kit (Pierce Chemical Co.) according to the manufacturer's instructions. Linker peptide was incubated with 9 mM sulfo-NHS-LC-biotin in PBS for 1 h at room temperature (RT). During biotinylation, vector/RBL cells, FPR/RBL cells, FPRL1/RBL cells, or HUVECs were trypsinized, collected, and treated with rabbit serum for 30 min at RT. After incubation with biotinylated linker peptide for 30 min on ice, the cells were briefly washed on ice with ice-cold PBS, incubated with antihuman fluoroscein-5-isothiocyanate (FITC)-conjugated streptavidin (Pierce Chemical Co.) for 30 min on ice, washed with ice-cold PBS, and fixed with a 1% formaldehyde solution. They were then analyzed using a FACS Caliber system (BD Biosciences, San Jose, CA) with CellQuest and WinMDI 2.9 software, as described previously [36].

2.8. Ca^{2+} measurement

Cells were incubated with fluo-3-AM working solution (Molecular Probes) [13] containing 0.03% pluronic F-127 (the final concentration of fluo-3-AM was 20 μM/L) for 1 h at 37 °C. After incubation, fluo-3-AM fluorescence in the cells was elicited at 488 nm with a high-power Ar^+ laser, and the emission bands were detected at 530 nm with a photomultiplier. The fluorescence signal was detected using a confocal laser scanning system (LSM 510 Meta; Carl Zeiss, Jena, Germany), equipped with a Nikon E-600 Eclipse microscope. The fluorescence intensity was measured both before (F_0) and after (F) the addition of CTGF. The change in intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ was expressed in terms of the F/F_0 ratio. A total of 50–120 images were scanned from each cell.

2.9. In vitro angiogenesis assay

For the proliferation assay, HUVECs were plated onto gelatin-coated-24-well culture dishes at a density of 2×10^4 cells/well and permitted to attach overnight. After 4 h of serum starvation, the cells were treated for 48 h with various mitogens. [^{3}H]-Thymidine (1 μCi, Amersham International) was added to each of the wells just before the final 6 h