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VEGF-A induces its negative regulator, soluble form of VEGFR-1, by modulating its alternative splicing

Tetsuya Saito [a], Norihiko Takeda [a,b,*], Eisuke Amiya [a], Tomoko Nakao [a], Hajime Abe [a], Hiroaki Semba [a], Katsura Soma [a], Katsuhiro Koyama [a], Yumiko Hosoya [a], Yasushi Imai [a], Takayuki Isagawa [a], Masafumi Watanabe [a], Ichiro Manabe [a], Issei Komuro [a,c], Ryozo Nagai [a,d], Koji Maemura [a,e,*]

[a] Department of Cardiovascular Medicine, Graduate School of Medicine, University of Tokyo, Japan
[b] PRESTO, JST, Japan
[c] Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Japan
[d] Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi 329-0498, Japan
[e] Department of Cardiovascular Medicine, Nagasaki University Graduate School of Biomedical Sciences, Japan

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ABSTRACT

Vascular endothelial growth factor-A (VEGF-A) is one of the major angiogenic factors, and its actions are primarily mediated through its two membrane receptors, VEGFR-1 and VEGFR-2. A soluble form of VEGFR-1 (sVEGFR-1) sequesters the free form of VEGF-A, and acts as a potent anti-angiogenic factor. While sVEGFR-1 is synthesized as a splice variant of VEGF-R1 gene, the interactions between VEGF-A and sVEGFR-1 remain largely unknown. Here, we show that VEGF-A upregulates sVEGF-R1 expression in human vascular endothelial cells but leaves full-length VEGF-R1 expression unchanged, and that this induction was dependent on the VEGFR-2-protein kinase C-MEK signaling pathway. The VEGF-A-induced sVEGFR-1 upregulation can operate as a negative feedback system, which if modulated can become a novel therapeutic target for regulating pathological angiogenesis.

1. Introduction

Vascular endothelial growth factor-A (VEGF-A) is a potent vascular endothelial cell (EC)-specific mitogen that stimulates EC proliferation, migration and angiogenesis, and it has also been shown to improve EC function and survival in vitro as well as vascular reactivity in vivo [1–3]. The actions of VEGF-A are primarily mediated through its two major tyrosine kinase receptors, namely VEGF receptor-1 (VEGFR-1, also known as fms-like tyrosine kinase; Flt1) and VEGF receptor-2 (VEGFR-2, also known as kinase insert domain receptor; KDR) [4]. Of these two receptors, VEGFR-2 possesses stronger tyrosine kinase activity and thus mediates most of the mitogenic signals of VEGF-A [1,5]. However, recent gene-targeting studies indicated VEGFR-1-mediated signalings have also significant roles in pathological angiogenesis [6]. Indeed, we previously reported that HIF-2α, a member of hypoxia-inducible transcription factors dominantly expresses in vascular endothelial cells, transactivates VEGFR-1 and enhances mature angiogenesis [7].

The soluble form of VEGFR-1 (sVEGFR-1) is transcribed as a splice variant of full-length VEGFR-1 by a premature polyadenylation of the VEGFR-1 transcript at the intron 13 [8]. Although soluble VEGFR-1 strongly binds to VEGF-A and placental growth factor (PlGF), it actually functions as a potent angiogenic inhibitor and causes endothelial dysfunction since it lacks the seventh immunoglobulin-like domain as well as the transmembrane and tyrosine kinase regions [9–12].

In cases of pathological angiogenesis such as preeclampsia and malignancies, sVEGFR-1 can potentially be used as a therapeutic target or as a prognostic biomarker. For example, hypoxic conditions of the placenta in preeclampsia not only increase VEGF-A expression, but also induces increased sVEGFR-1 secretion. The resultant net effect is a reduced bioavailability of free VEGF-A and PlGF, thereby leading to hypertension, glomerular dysfunction and proteinuria [13–16]. Indeed, increased levels of sVEGFR-1 and reduced levels of PlGF have been demonstrated to be predictors of the subsequent development of preeclampsia [17]. Moreover, a

* .Corresponding authors Address: Department of Cardiovascular Medicine, Graduate School of Medicine, University of Tokyo, Japan.
E-mail address: ntakeda-tky@umin.ac.jp (N. Takeda).

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lower sVEGFR-1/VEGF-A or sVEGFR-1/PlGF ratio correlates significantly with a more favorable prognosis and better responsiveness to anti-cancer therapy in gliomas, breast cancer and pancreatic cancer patients [18–21]. These results indicate that angiogenesis is strictly regulated by the balance between pro- and anti-angiogenic factors, and that any imbalances between them have the potential to elicit pathological angiogenic disorders.

The phenomenon of feedback interactions between a ligand and its endogenous inhibitor, such as seen with Wnt glycoproteins and its antagonists during processes of cell proliferation and differentiation, has been extensively reported elsewhere. Wnt signaling is negatively regulated by soluble antagonists such as secreted Frizzled-related protein-1 (sFRP-1) or DICKKOPF-1 [22], the expressions of which are in fact induced by canonical Wnt signaling and thereby creates a negative feedback loop to antagonize persistent Wnt signal activation under physiological conditions [23,24].

In a similar way, we therefore hypothesized that sVEGFR-1 may act as a negative regulator in VEGF-A signaling. In this study, we identified that VEGF-A, but not PlGF, affects the splicing processes of the VEGFR-1 gene via the VEGFR-2-protein kinase C (PKC)-MEK pathway, leading to the induction of sVEGFR-1 expression. The induction of sVEGFR-1 splicing is dependent on the premature polyadenylation of its intron 13 [8]. We also confirmed that a VEGF-A responsive element exists within intron 13 of the VEGFR-1 gene.

Our findings give insights into a novel negative feedback system in physiological or pathological angiogenesis that, if modulated, may be a novel therapeutic target in the treatment of numerous pathological angiogenic disorders.

2. Materials and methods

2.1. Materials

Human recombinant VEGF-A, human recombinant PlGF, and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were obtained from R&D Systems (Minneapolis, MN). CalphostinC, u0126, LY294002 and SB203580 were obtained from Calbiochem (La Jolla, CA).

2.2. Cell culture

Human aortic endothelial cells (HAECs), human umbilical vein endothelial cells (HUVECs), and human aortic smooth muscle cells (hASMCs) were obtained from BioWhittaker (Walkersville, MD). Human aortic endothelial cells and HUVECs were maintained in endothelial growth medium 2 (EGM-2, Cambrex) while HASMCs were maintained in smooth muscle growth medium 2 (SMGM-2, Cambrex). AD293 cells (human embryonic kidney cells), bEnd.3 cells (murine brain endothelial cells), EOMA cells (murine hemangioendothelioma endothelial cells) and RAW cells (murine macrophage cells) were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich JAPAN, Tokyo, Japan) containing 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin. All cells were cultured at 37 °C, 5% CO_2 and cells from passages 5 to 10 were used for the experiments.

2.3. Northern blot analysis

Total cellular RNA was extracted by the RNeasy Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. Northern blot analysis using probe 1 was performed as described previously [7]. The 350 bp (AL138712 bp157156–157505) fragment of the proximal end of intron 13, the 465 bp (AL138712 bp153527–153991) fragment of the distal end of intron 13, and the 436 bp (AL138712 bp152304–152739) fragment of exon 14 of the human VEGFR-1 gene were amplified by PCR with, respectively, VEGFR-1 probes 2, 3, and 4 (see Fig. 1A). To correct for differences in RNA loading, the membranes were rehybridized with radiolabeled 18S oligonucleotide.

2.4. Western blot analysis

Total cellular protein of HAECs was extracted using extraction buffer (25 mM Tris, pH 7.4, 50 mM NaCl, 0.5% Na deoxycholate, 2% NP-40, and 0.2% SDS). Equal amounts of protein (10 μg) were subjected to SDS–PAGE as described previously [7]. Full-length VEGFR-1 and sVEGFR-1 were detected using monoclonal anti-hVEGFR-1 mouse IgG antibody (Sigma–Aldrich, 1:1000) and anti-mouse horseradish peroxidase-conjugated IgG (Cell Signaling, 1:5000) as a second antibody, followed by enhanced chemiluminescence plus detection (GE healthcare Japan, Tokyo, Japan).

2.5. siRNA duplex and transfection

The siRNA directed against full-length *VEGFR-1* was designed to target a sequence in Exon 27 of the VEGFR-1 gene, and the sequences of the siRNA duplex were 5′-UUGGGAUGUAGUCUUUACCdTdT-3′ (sense strand) and 5′-GGUAAAGACUACAUCCCAAdTdT-3′ (antisense strand). The scrambled control duplex did not target any gene, and the sequences were 5′-CUUACUAACAUGGUGUGUUdTdT-3′ (sense strand) and 5′-AACACACCAUGUUAGUAAGdTdT-3′ (antisense strand). The siRNA duplexes were synthesized using the Silencer siRNA Construction Kit (Ambion) according to the manufacturer's instructions. The siRNA directed against *VEGFR-2* was purchased from Dharmacon (Target sequences: GGGCAUGUACUGACGAUUA, CUACAUUGUUCUUCCGAUA, GGAAAUCUCUUGCAAGCUA and GCGAUGGCCUCUUCUGUAA).

HAECs were plated onto 6-cm cell culture dishes and grown to 70% confluence before transfection by adding a total of 10 μl of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) and 200 pmol siRNA duplex.

2.6. Construction of reporter plasmids

The pGL3-Basic plasmid was purchased from Promega (Madison, WI). To generate the VEGFR-1 reporter plasmid (Fig. 4-A-(1)), the promoter region of the VEGFR-1 gene from position −1160 to +305 was amplified by PCR and subcloned into the pGL3-Basic plasmid between *Sac*I and *Hind*III restriction sites. To generate the reporter plasmid with a modulated poly(A) signal, the intron 13 fragment (AL138712 bp152676–157553) of the VEGFR-1 gene was amplified by PCR and inserted into the VEGFR-1 reporter plasmid between *Sal*I and *Xba*I restriction sites in place of the SV40 late poly(A) signal (Fig. 4-A-(2)).

2.7. Transient transfection assay

HAECs were cultured in 12-well culture plates and transfected with 2.0 μg of the reporter construct using the Neon Transfection system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To correct for variations in transfection efficiency, we cotransfected 0.1 μg of pCMV-βgal in all experiments. Cell extracts were prepared by a detergent lysis method (Promega KK, Tokyo, Japan). The ratio of luciferase activity to β-galactosidase activity in each sample served as a measure of normalized luciferase activity. Each construct was transfected at least 3 times, and each transfection was done in triplicate.

2.8. Statistical analysis

Results are expressed as mean ± S.E. Two-tailed Student's *t* test for unpaired samples or one-way ANOVA was used for parameter comparisons, and a $P < 0.05$ was considered significant.