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Research paper

Depletion of the novel protein PHACTR-1 from human endothelial cells abolishes tube formation and induces cell death receptor apoptosis

Rafika Jarray [a,2], Barbara Allain [a,b,2], Lucia Borriello [a,b], Denis Biard [c], Ali Loukaci [a], Jérôme Larghero [d,e,f], Réda Hadj-Slimane [b], Christiane Garbay [a], Yves Lepelletier [g,1,3], Françoise Raynaud [a,*,3]

[a] Université Paris Descartes, Sorbonne Paris Cité, CNRS UMR 8601, Laboratoire de chimie et biochimie pharmacologiques et toxicologiques, 45 rue des Saints-Pères, 75006 Paris, France
[b] Tragex Pharma, 34 rue Blomet, 75015 Paris, France
[c] Commissariat à l'Energie Atomique, DSV-iRCM, 92265 Fontenay aux Roses Cedex 6, France
[d] AP-HP, Hôpital Saint-Louis, Unité de Thérapie Cellulaire et CIC de Biothérapie, Paris, France
[e] Université Paris Diderot, Sorbonne Paris Cité, F-75475 Paris, France
[f] INSERM UMR940, Institut Universitaire d'Hématologie, Hôpital Saint-Louis, Paris, France
[g] Université Paris Descartes, Sorbonne Paris Cité 149-161 rue de Sèvres, 75015 Paris, France

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ABSTRACT

Using suppression subtractive hybridisation (SSH), we identified a hitherto unreported gene *PHACTR-1* (Phosphatase Actin Regulating Protein-1) in Human Umbilical Vascular Endothelial Cells (HUVECs). PHACTR-1 is an actin and protein phosphatase 1 (PP1) binding protein which is reported to be highly expressed in brain and which controls PP1 activity and F-actin remodelling. We have also reported that its expression is dependent of Vascular Endothelial Growth Factor (VEGF-A_{165}). To study its function in endothelial cells, we used a siRNA strategy against *PHACTR-1*. PHACTR-1 siRNA-treated HUVECs showed a major impairment of tube formation and stabilisation. PHACTR-1 depletion triggered apoptosis through death receptors DR4, DR5 and FAS, which was reversed using death receptor siRNAs or with death receptor-dependent caspase-8 siRNA. Our findings suggest that PHACTR-1 is likely to be a key regulator of endothelial cell function properties. Because of its central role in the control of tube formation and endothelial cell survival, PHACTR-1 may represent a new target for the development of anti-angiogenic therapy.

1. Introduction

Angiogenesis is a fundamental process occurring during embryonic [1] and adult life [2], resulting in the formation of new blood vessels from existing vascular beds. It requires the synchronised action of several different growth factors in endothelial cells. This complex process is closely regulated and controlled by balance between negative (anti-angiogenic) and positive (pro-angiogenic) factors [3,4]. The deregulation of angiogenesis by disruption of this balance induces a number of vascular-dependent diseases such as tumour growth and metastasis, as well as inflammatory and immune diseases [1].

Among the numerous pro- and anti-angiogenic factors, VEGF is a key regulator of developmental angiogenesis as loss of a single VEGF allele results in embryonic lethality. The VEGF pathway also plays an essential role in reproductive and bone angiogenesis. In mammals, the VEGF family comprises five members including VEGF-A (hereafter called VEGF), VEGF-B, VEGF-C, VEGF-D and PlGF (placenta growth factor). Alternative exon splicing results in generation of several VEGF isoforms including $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ [5–7].

VEGF-A was first known as vascular permeability factor and is a potent pro-angiogenic factor [8–10]. VEGF-A_{165} is over-expressed in diseases involving excess angiogenesis such as age-related macular degeneration and diabetic retinopathy [11] and in cancers such as melanoma and colorectal carcinoma [7]. VEGF-A_{165} acts through its membrane receptors, VEGF-R and neuropilins [12–14]. Only the pro-angiogenic form of VEGF-A_{165} has the basic amino acids to bind neuropilin-1 (NRP-1) [15] and neuropilin-2 (NRP-2), the latter with lower affinity than NRP-1.

Molecular biomarker research in angiogenesis inhibition is an actively growing field. Although current data are extremely promising, it is still uncertain which biomarkers can reliably predict the efficacy of anti-angiogenic therapy. With increasing numbers of

* Corresponding author. Tel.: +33 1 42 86 20 60; fax: +33 1 42 86 40 82.
E-mail address: francoise.raynaud@parisdescartes.fr (F. Raynaud).
[1] Present address: 24 rue Docteur Calmette, 91200 Athis-Mons, France.
[2] These authors contributed equally to this work.
[3] These authors co-directed this work.

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inhibitors being developed, the need for biomarkers is more critical than ever for clinical use in human disease management and knowledge.

We sought to determine functional targets of VEGF. By using Suppression Subtractive Hybridisation (SSH), we describe for the first time *PHACTR-1* as a VEGF-A_{165}-induced gene expressed in human primary endothelial cells. We investigate the effects of PHACTR-1 depletion in human endothelial cell (HUVECs) by using a siRNA approach. Disruption of PHACTR-1 dramatically reduced tube formation and triggered induction of the extrinsic apoptotic pathway.

2. Materials and methods

2.1. Cell culture

Primary human umbilical vein endothelial cells (HUVECs) (Lonza, Belgium) were cultured in the presence of EGM-2 medium complemented with EBM-2 growth factor mix, supplemented with 2% SVF (Lonza, Belgium) at 37 °C, 5% CO_2. Only cells from passages 2–6 were used for experiments.

2.2. Suppression subtractive hybridisation (SSH)

SSH was performed with a PCR-SELECT cDNA subtraction kit (Clontech, France) following the manufacturer's instruction. A 4-fold greater than recommended amount of driver cDNA was added to the second hybridisation. Starting material consisted of HUVEC mRNA as "tester" and a pool of 8 non-umbilical vein endothelial cell lines including epithelial tumour cells (MCF7, CHA, HeLa, Wish, U373) and lymphoblasts (Jurkat, Daudi, U266) mRNA as "driver". Thirty primary PCR cycles and 12 secondary PCR cycles were performed.

2.2.1. Cloning and sequencing of cDNAs

PCR products generated by SSH were subcloned into PCR 2.1 vector using the original TA-cloning kit (Invitrogen, France). Subcloned cDNAs were isolated by colony PCR amplification. Sequencing was performed using an automated ABI-370A-DNA sequencing system. Sequence reactions were carried out with an ABI prism dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, France). The sequences obtained were compared using GenBank™/EBI and expressed sequence tag data bases using BLAST searches.

2.2.2. Construction of subtracted cDNA library

HUVECs and $VEGF_{A165}$-treated HUVECs were homogenised in a denaturing solution containing 4 M guanidium thiocyanate. After sonication, lysate was centrifuged using discontinuous CsCl density (2.4 and 5.7 M) gradient, the RNA pellet was dissolved and extracted with phenol/chloroform. Poly(A) RNA was prepared by three rounds of affinity chromatography on oligo(dT)-cellulose (Amersham Biosciences, France). These two mRNA populations were compared by SSH using a Clontech PCR-Select cDNA Subtraction Kit (BD Biosciences, USA). Tester and driver cDNAs were digested with *Rsa* I. Tester cDNA was then subdivided into two parts, and ligated with different cDNA adaptors (adaptors 1 and 2R). Two hybridisations were performed. In the first, an excess of driver was added to each tester sample. Samples were then heat denatured and allowed to anneal. During the second hybridisation, the two primary hybridisation samples were mixed together without denaturing. Only the remaining single-strand tester cDNA can re-associate. These hybrids are double-stranded tester molecules with different ends, which correspond to adaptor 1 and 2R sequences. Fresh denatured driver cDNA was added (without denaturing the subtraction mix) to further enrich each fraction for differentially expressed sequences. The entire population of molecules was then subjected to PCR (25 cycles) to amplify the desired differentially expressed sequences. Only molecules that have two different adaptors can be amplified exponentially. A secondary PCR amplification (20 cycles) was performed to further reduce any background PCR products and to enrich for differentially expressed sequences. cDNAs were then inserted directly into a T/A cloning vector (pGEM-T) (Promega, USA) to make a subtracted cDNA library. cDNA clones were sequenced and identified using BLAST searches (GenBank/EMBL database). Two subtractions were performed: the principal subtraction (forward subtraction) and a reverse subtraction in which tester serves as the driver and the driver as tester.

2.3. Cell viability assay

The exponentially growing cells were seeded at 3000 cells per well in 96-well plates. After 24 h, they were treated with scramble siRNA and PHACTR-1 siRNA at 10 nM. After 72 h, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1; Roche®) was added and cells were incubated at 37 °C for 1–2 h. Optical density was measured with a microplate reader (Bio-Rad) at 490 nm to determine cell viability.

2.4. Cell proliferation assay

72 h after transfection, cells were trypsinised and resuspended in complete medium. Each sample was mixed in trypan blue (0.14% in HBBS). Coloured (non-viable) and dye-excluding (viable) cells were counted in a Malassez hemocytometer.

2.5. RNA purification and cDNA synthesis

HUVEC RNA was extracted with NucleoSpinRNA II (Macherey-Nagel, France) and quantified using Nanodrop (ND-1000 spectrophotometer). 1 μg of each RNA sample was reverse-transcripted into cDNA using an iScript cDNA Synthesis Kit (Bio-Rad, USA) with 20 μL of random hexamer primers, oligo-dT mixes and incubated at 25 °C for 5 min, then at 42 °C for 30 min and at 85 °C for 5 min.

2.6. Real-time quantitative PCR (RT-qPCR)

Relative gene expression level of *PHACTR-1* gene in depleted HUVECs was determined by real-time quantitative PCR (RT-qPCR). The sequences of primers used were for *PHACTR-1*: 5′-GAG-GCA-AAG-CAG-AGA-AGA-GC-3′ and 5′-CAT-GAT-GTC-TGA-CGG-TTG-GA-3′. Ribosomal protein *RPLO* was used as a reference gene, primers used were: 5′-CAT-TGC-CCC-ATG-TGA-AGT-C-3′ and 5′-GCT-CCC-ACT-TTG-TCT-CCA-GT-3′. Two μL of cDNA (1:5 diluted) and 0.3 μM primers were mixed with components from the IQ™ SYBR® Green Supermix kit (Bio-Rad, France) in a final volume 10 μL. Reactions in triplicate were carried out in the MiniOpticon real-time PCR machine (Bio-Rad, France) under the following conditions: initial denaturation at 95 °C for 3 min and then 45 cycles of denaturation at 95 °C for 10 s, annealing/extension at 60 °C for 30 s. Melting curves were obtained to examine the purity of amplified products. Absolute quantitative data and Cq values were obtained by analysis with Bio-Rad MFX Software 2.0 by the second derivative method. Data normalisation was done as follows: [copy numbers *PHACTR-1* in a sample/copy numbers of reference gene *RPLO* in a sample)]/[copy numbers *PHACTR-1* in the control/copy numbers of reference gene *RPLO* in the control]. The *p*-values were obtained by independent ANOVA.