Neurophilin-1 is a downstream target of transcription factor Ets-1 in human umbilical vein endothelial cells

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Abstract Transcription factor Ets-1 expressed in endothelial cells promotes angiogenesis. Here, we transiently overexpressed Ets-1 in human umbilical vein endothelial cells (HUVECs) and comprehensively searched for potential downstream targets of Ets-1 by cDNA microarray analysis. The expression of several angiogenesis-related genes including neuropilin-1 was augmented by the overexpression of Ets-1. Quantitative real-time RT-PCR and Western blotting confirmed the increase in the levels of neuropilin-1 mRNA and protein. In contrast, dominant negative ets-1 decreased the levels of neuropilin-1 mRNA and protein. These results indicate that neuropilin-1 is a downstream target of Ets-1 in HUVECs.

Key words: Endothelial cell; Ets-1; Neuropilin-1; Angiogenesis

1. Introduction

Angiogenesis, the formation of neo-vessels from pre-existing ones, occurs in a wide range of physiological as well as pathological states, including embryogenesis, wound healing, diabetic retinopathy, rheumatoid arthritis, and solid tumors. Angiogenesis is regulated by the balance between angiogenic factors and angiogenesis inhibitors. Numerous molecules have been reported to regulate angiogenesis. Among them, vascular endothelial growth factor (VEGF) is the most important angiogenic factor. VEGF binds to two signal-transducing receptor-type tyrosine kinase, Flt-1 (VEGFR-1) and KDR/Flk-1 (VEGFR-2), and stimulates protease synthesis, migration and proliferation of endothelial cells (ECs) [1]. More recently, the semaphorin receptor neuropilin-1 was identified as the binding site of VEGF on the surface of ECs [2]. Neuropilin-1 is thought to accumulate VEGF on the cell surface and transfer it to signal-transducing VEGF receptors.

ECs express various genes during angiogenesis, and thus an understanding of the transcriptional control of these genes in ECs is important. Ets-1 is a prototype of the Ets family of transcription factors. Ets-1 is expressed in ECs during angiogensis or re-endothelialization after denuding injury [3,4]. Angiogenic factors including VEGF are potent inducers of Ets-1 in ECs [3,5,6]. In contrast, TGF-β attenuates the transactivation activity of Ets-1 by inhibiting the binding of Ets to DNA via Ets binding motif [7]. Ets-1 positively regulates angiogenesis [3,8], and the elimination of the effect of Ets-1 by a dominant negative molecule inhibits angiogenesis in vivo [9]. The genes for several molecules including uPA, MMP-1, MMP-3, MMP-9, integrin β3, and VE-cadherin are reported to be downstream targets of Ets-1 in ECs [3,8,10]. Moreover, endothelium-specific, receptor-type tyrosine kinase such as Flt-1, KDR, TIE-1 and TIE-2 contain the Ets binding motif in their promoter/enhancer regions, and the Ets family transcription factors are suggested to stimulate their promoter activities [11–14].

To further clarify the downstream targets of Ets-1 in ECs, we transiently overexpressed Ets-1 in human umbilical vein endothelial cells (HUVECs) with an adenovirus vector encoding the wild-type *ets-1* gene (Adets-1) and comprehensively searched for potential downstream targets by cDNA microarray analysis. Our analysis revealed that neuropilin-1 is yet another downstream target of Ets-1 in ECs.

2. Materials and methods

2.1. Cell culture

HUVECs were obtained from Kurabo (Osaka, Japan), and cultured on type-I collagen-coated dishes (Iwaki, Tokyo, Japan) in endothelial basal medium (EBM) containing EC growth supplements (Clonetics Corp., Walkersville, MD, USA) and 10% fetal bovine serum (FBS, JRH Biosciences, San Antonio, TX, USA).

2.2. Adenovirus-mediated gene transfer

Adenovirus vectors encoding wild-type *ets-1* (Adets-1), transdominant mutants *ets-1* (AdTMets-1), and a control adenovirus (Adnull) were described elsewhere [9,15]. HUVECs were infected with the adenovirus vectors at a multiplicity of infection (m.o.i.) of various values. Adenoviral infection was carried out in agitated serum-free M199 (Nissui, Tokyo, Japan) for 1 h at 37°C, and the medium was then changed to M199 containing 20% FBS, after which the cells were further incubated for the desired periods of time.

2.3. cDNA microarray analysis

Total RNA was prepared from HUVECs infected with Adets-1 or Adnull by use of ISOGEN (Nippongene, Toyama, Japan), and poly(A)+ RNA was purified from the total RNA by using an Oligotex-dT30 ⟨super⟩ mRNA purification kit (Takara Shuzo Co., Ltd, Shiga, Japan). cDNA probe synthesis, hybridization with a human UniGEM V cDNA microarray, and signal analysis were conducted by Genome Systems (St. Louis, MO, USA). Clones with a Cy3 signal/Cy5 balanced signal ratio larger than 2.5 were considered to be induced.

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2.4. Quantitative RT-PCR analysis

Total RNA samples were prepared from subconfluent cultures of HUVECs infected with Adets-1, AdTMets-1 or Adnull by use of ISOGEN. First-strand cDNAs were generated with a 1st Strand cDNA Synthesis kit for RT-PCR (Roche Diagnostics, Mannheim, Germany). Quantitative RT-PCR was conducted with a LightCycler™ and SyberGreen System (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction. The human neuropilin-1 primer set and the hybridization probes used were the following: sense 5′-CCAAGCTTTTCTCAGGAGCTTCA-3′ and antisense 5′-TTTC-CGTGAACTCTCCTGTA-3′.

2.5. Western blot analysis

Cells were extracted with modified RIPA buffer (2 mM sodium orthovanadate, 50 mM NaF, 20 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 5 mM sodium pyrophosphate, 10% glycerol, 0.2% Triton X-100, 5 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Equal amounts of samples were applied for SDS–PAGE under reducing conditions and transferred to a nitrocellulose membrane (Hybond ECL, Amersham, Buckinghamshire, England). The membrane was incubated with rabbit polyclonal anti-human neuropilin-1 antibody (Santa Cruz Biotechnology, Inc., CA, USA) as a primary antibody according to the manufacturer's instructions. The signal was visualized by using HRP-conjugated secondary antibodies and enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, England) with an LAS-1000 image analyzer (Fuji, Tokyo, Japan).

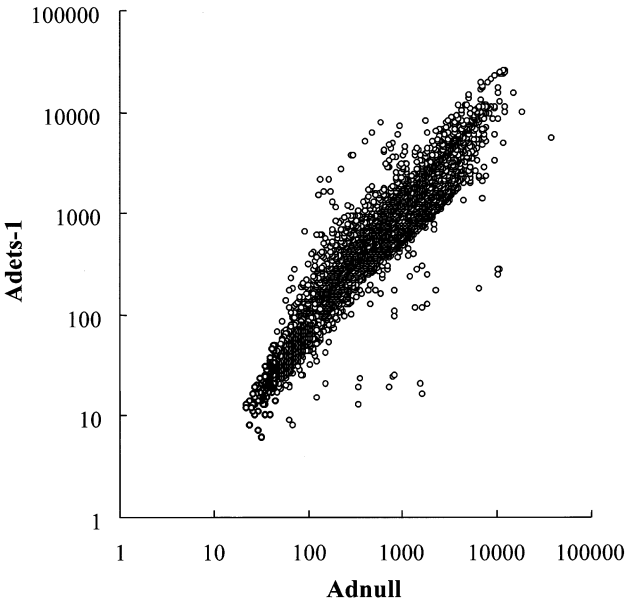

Fig. 1. Expression profile of HUVEC genes analyzed with a cDNA microarray. HUVECs were infected with Adnull or Adets-1. After a 48-h incubation, poly(A)+ RNA was obtained. Microarray analysis was performed as described in Section 2. The scatter plot of expression in Adets-1-infected HUVECs versus that in Adnull-infected ones is shown.

3. Results

To explore the downstream targets of Ets-1 in ECs, we infected HUVECs with Adets-1 and compared the expression profile with that of HUVECs infected with Adnull. After a 48-h incubation, we analyzed the expression profile with a cDNA microarray. The scatter plot of expression in Adets-1-infected HUVECs versus that in Adnull-infected ones is shown in Fig. 1. A number of genes were modulated in their expression. Among the 9375 human genes in the microarray, 110 of them were augmented in their expression more than 2.5 fold.

Twenty-two of the known angiogenesis-related genes in the present microarray were selected and their relative expression are shown in Table 1. It appeared that the expression of seven genes was augmented more than 2.5 folds by the exogenously expressed Ets-1 in HUVECs. Of these, MMP-1 is the only molecule that has already been recognized as a downstream target of Ets-1 in ECs. The other six genes, including neuropilin-1, have not been previously recognized as downstream targets of Ets-1.

Neuropilin-1 is the binding site of VEGF on the surface of ECs, and is thought to modulate the effect of VEGF on ECs. However, transcriptional regulation of its expression is obscure at the moment. Therefore, we clarified the expression of neuropilin-1 by real-time RT-PCR analysis. A time course experiment revealed that an increase in the level of neuropilin-1 mRNA was evident as early as 24 h after the infection (Fig. 2A). This increase was detected at an m.o.i. as low as 10, and was maximal at an m.o.i. of 100 (Fig. 2B). To further prove the involvement of endogenous Ets-1 in the expression of neuropilin-1, we used the adenovirus encoding transdominant mutant Ets-1 (AdTMets-1), which acts as a dominant negative

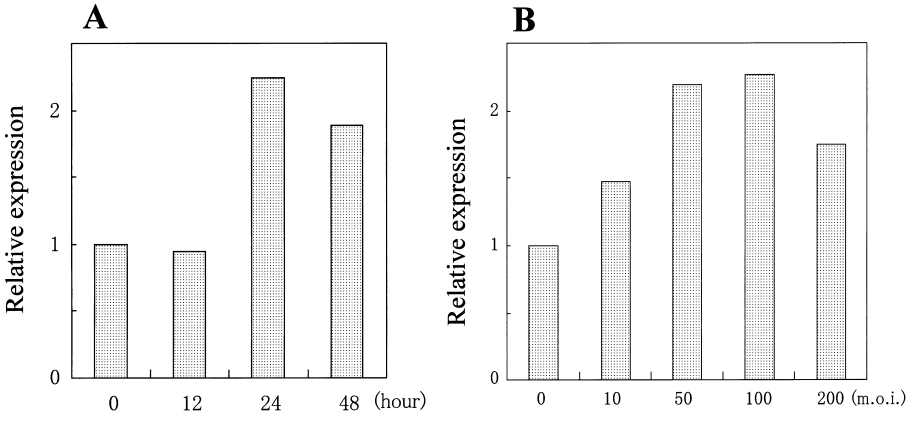

Fig. 2. Time course and m.o.i. dependency of Adets-1 on the expression of neuropilin-1 mRNA in HUVECs. A: HUVECs were infected with 200 m.o.i. of Adnull or Adets-1. At the indicated time points, total RNA was obtained, and real-time RT-PCR was then performed. B: HUVECs were infected with the indicated m.o.i. of Adnull or Adets-1. After a 48-h incubation, total RNA was obtained, and real-time RT-PCR was performed. The expression of neuropilin-1 in Adets-1-infected HUVECs was standardized with that in Adnull-infected HUVECs.

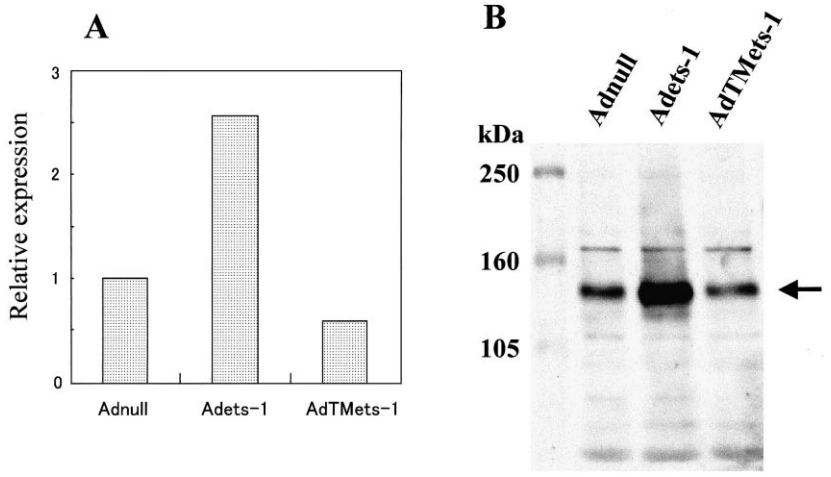

Fig. 3. Effect of dominant negative ets-1 on the expression of neuropilin-1 in HUVECs. A: HUVECs were infected with the indicated m.o.i. of Adnull, Adets-1 or AdTMets-1. After a 48-h incubation, total RNA was obtained, and real-time RT-PCR was then performed. Neuropilin-1 mRNA level in the Adnull-infected HUVECs was expressed as 1. B: HUVECs were infected with the indicated m.o.i. of Adnull, Adets-1 or AdTMets-1. After a 48-h incubation, protein was extracted, and Western blotting for neuropilin-1 was performed.

over Ets-1. The real-time RT-PCR analysis showed that AdTMets-1 decreased the level of neuropilin-1 mRNA in the same experiment where Adets-1 increased it (Fig. 3A). Moreover, AdTMets-1 decreased the protein level of neuropilin-1 as well (Fig. 3B).

4. Discussion

The cDNA microarray analysis revealed that Ets-1 augmented the expression of seven angiogenesis-related genes in HUVECs. Among them, six genes have not been previously recognized as downstream targets of Ets-1. We further clarified one of those genes, namely, neuropilin-1. Neuropilin-1 was initially identified as a membrane protein that is expressed in developing neurons, and it was found to function as a receptor for the class 3 semaphorins, which produce inhibitory axon guidance signals [16]. More recently, neuropilin-1 was shown to be expressed in ECs and to function as a co-receptor for $VEGF_{165}$ [2] and several other members of the VEGF family [17,18]. The neuropilin-1 mutant mouse embryos exhibited severe abnormalities in their nervous as well as cardiovascular system [19,20], indicating that neuropilin-1 plays important roles in embryonic nervous tissue and vessel formation. Neuropilin-1 is thought to be involved in postnatal angiogenesis as well, since the expression of neuropilin-1 correlates with that of VEGFR2 in the tissues of rheumatoid arthritis and diabetic proliferative retinopathy [21,22].

Table 1
The expression profile of angiogenesis-related genes in HUVECs

Gene name	Fold induction
podocalyxin-like	3.3
ephrin-A1	3.1
MMP-1	3.0
angiopoietin-2	2.7
calreticulin	2.7
neuropilin-1	2.6
uPA receptor	2.5
FGFR-1	2.4
MMP-2	2.3
VE-cadherin	1.7
integrin β3	1.3
Flt-1	1.3
TIE-1	1.2
Eph B4	1.2
ephrin B2	1.1
uPA	1.1
KDR	0.9
MMP-9	0.8
TIMP-1	0.8
MMP-14 (MT1-MMP)	0.7
angiopoietin-1	0.6
bFGF	0.4

Although neuropilin-1 is recognized as an important mediator of angiogenesis, information on the transcriptional regulation of the neuropilin-1 gene in ECs is limited. Targeted deletion of the dHAND gene in mice results in severe defects of embryonic and yolk sac vascular development and the downregulation of neuropilin-1 in ECs [23]. However, since the expression of dHAND is restricted to smooth muscle cells, the downregulation of neuropilin-1 in ECs does not directly relate to targeted deletion of the dHAND gene. Our present results suggest for the first time that neuropilin-1 is the downstream target of Ets-1 in ECs. Tumor necrosis factor-α (TNF-α) was shown to induce the expression of both neuropilin-1 [24] and Ets-1 [25] in ECs. Thus, TNF-α may induces the expression of neuropilin-1 via the induction of Ets-1. Further study is required to clarify the promoter region of neuropilin-1 gene to determine whether or not an Ets binding site(s) exists and functions there.

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