Vascular Endothelial Zinc Finger 1 Is Involved in the Regulation of Angiogenesis: Possible Contribution of Stathmin/OP18 as a Downstream Target Gene

Hiroki Miyashita, Masanori Kanemura, Tohru Yamazaki, Mayumi Abe, Yasufumi Sato

Objective—Vascular endothelial zinc finger 1 (Vezf1) is a recently identified zinc finger transcription factor that is expressed in endothelial cells (ECs) during vascular development in mouse embryo. Here, we present that Vezf1 was expressed in ECs at the site of postnatal angiogenesis. We therefore examined whether Vezf1 was involved in the regulation of angiogenesis.

Methods and Results—The specific downregulation of Vezf1 by antisense oligodeoxynucleotide (AS-ODN) significantly inhibited the proliferation, migration, and network formation of cultured ECs as well as angiogenesis in vivo. Vezf1 AS-ODN downregulated the expression of stathmin/oncoprotein18 (OP18), a microtubule-destabilizing protein, in ECs, whereas transient transfection of Vezf1 cDNA increased the expression of stathmin/OP18 in ECs. To explore the relationship between Vezf1 and stathmin/OP18, we specifically downregulated stathmin/OP18. We found that stathmin/OP18 AS-ODN inhibited the proliferation, migration, and network formation of ECs as Vezf1 AS-ODN did. Moreover, Vezf1 AS-ODN decreased G2/M population of ECs and increased apoptosis, which reproduced the characteristic feature of stathmin/OP18 inhibition.

Conclusion—These results suggest that Vezf1 is involved in the regulation of angiogenesis, at least in part, through the expression of stathmin/OP18 in ECs. (Arterioscler Thromb Vasc Biol. 2004;24:878-884.)

Key Words: Vezf1 • transcription factor • endothelial cell • angiogenesis • stathmin/OP18

The vascular system is the first functional organ that forms in the embryo. At the first step of vascular formation, a subset of mesodermal cells, termed hemangioblasts, and/or angioblasts aggregate and differentiate to endothelial cells (ECs) and form the primary vascular plexus. This process is known as “vasculogenesis.” In the following process of “angiogenesis,” neovessels are generated from the primary vascular plexus by sprouting and/or intussusception and become distributed throughout the entire embryo. In the final process of vascular formation, mesenchymal cells surround the blood vessels and differentiate into mural cells (smooth muscle cells or pericytes) and make vessels mature and stabilized.1 In contrast, blood vessels in adulthood are composed of ECs and mural cells. Angiogenesis in adulthood commences with the detachment of preexisting mural pericytes from preexisting microvessels (vascular destabilization), followed by sprouting and/or intussusception of ECs, and finally vascular stabilization by reattachment of pericytes.2 A number of factors have been reported to act on ECs and regulate vascular formation in embryo or angiogenesis in adulthood. They include: vascular endothelial growth factor (VEGF)3,4 and its receptors fms-like tyrosine kinase 1 (Flt1)5 and fetal liver kinase 1 (Flk1);6 angiopoietins and their receptor tyrosine kinase with Ig-like loops and epidermal growth factor homology domain 2 (TIE2);7–9 and ephrinB2 and its receptor, EphB4.10,11

During the processes of vascular formation, a number of genes are expressed in ECs. Thus, the regulation of genes expression in ECs should be focused for understanding the molecular mechanism of vascular formation. Several transcription factors are reported to be expressed in ECs during vascular formation. They include E twenty-six 1 (ETS1),12 stem cell leukemia (SCL)/T-cell leukemia 1 (TAL1),13,14 translocated ETS leukemia (TEL),15 hematopoietically expressed homeobox (HEX),16 hypoxia inducible factor 1 (HIF1),17 and Friend leukemia integration-site 1 (Flt1).18 Vascular endothelial zinc finger 1 (Vezf1) belongs to zinc finger transcription factors that possess 6 (Cys2-His2)-type zinc finger motifs, whose expression is observed in the vascular endothelium in mouse embryo.19 Thus, Vezf1 is nominated as an additional candidate of transcription factor that may regulate vascular formation.

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From the Department of Vascular Biology, Institute of Development, Aging, and Cancer, Tohoku University, 4-1, Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan.
Present address of H.M.: University of Connecticut School of Medicine, Vascular Biology Center, Room E5024, 263 Farmington Avenue, Farmington, CT 06030.
Correspondence to Dr Yasufumi Sato, Department of Vascular Biology, Institute of Development, Aging, and Cancer, Tohoku University, 4-1, Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. E-mail y-sato@idac.tohoku.ac.jp © 2004 American Heart Association, Inc.

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Although mechanisms during vascular formation in embryonic and angiogenesis in adulthood may not be identical, there are strong evidences that they overlap and that molecules required for vascular development contribute to angiogenesis in adulthood as well. Here, we present that Vezf1 was expressed in ECs at the site of angiogenesis. We therefore examined whether Vezf1 played any roles in postnatal angiogenesis. Our results suggested that Vezf1 played a role in angiogenesis. The possible downstream target of Vezf1 in ECs was further discussed.

Methods

Materials

The following materials were used: VEGF (R&D Systems, Minneapolis, Minn); growth factor-reduced Matrigel (Collaborative Research, Bedford, Mass); α-minimal essential medium (αMEM), Opti-MEM, Lipofectin, Superscript II reverse transcriptase, and oligo(dT)12 to 18 primer (Invitrogen, Carlsbad, Calif); ribonuclease A, propidium iodide, and anti-oligo(dT)12 to 18 primer (Gibco BRL, Rockville, Md); ribonuclease A, propidium iodide, and anti-β-actin antibody (Ab) (Sigma, St. Louis, Mo); anti-von Willebrand factor (vWF) Ab and antistathmin/oncoprotein18 (OP18) Ab (Santa Cruz Biotechnology, Santa Cruz, Calif); anti-His6 Ab (Roche, Mannheim, Germany); Isogen (Nippon Gene, Toyama, Japan); and Hybond N’ membrane and nitrocellulose membranes (Amersham, Buckinghamshire, UK).

Cells

A mouse EC line MSS31,20 a spontaneously immortalized cell line from spleen ECs, was cultured in αMEM supplemented with 10% fetal calf serum (FCS) as described previously.21

Preparation of Vezf1 and Stathmin/OP18 cDNA

Full-length cDNAs of Vezf1 and Stathmin/OP18 were prepared by RT-PCR. cDNAs were generated from total RNA of MSS31 cells with Superscript II reverse-transcriptase and oligo(dT)12 to 18 primer and amplified with Advantage 2 PCR Enzyme system kit (Clontech, Palo Alto, Calif). The following primers were synthesized and used for amplification: for Vezf1, forward primer, 5’-ATGGAGGCCAAC-TGAATGAGGCA-3’; and reverse primer, 5’-CCAAGGGCTGT-GGTTACCTGC-3’; and for Stathmin/OP18, 5’-ATGGCTTCTCTCT-ATATCCA-3’; and reverse primer, 5’-TTATGTCAGGTCTAGTCTC-3’. Polymerase chain reaction products were inserted into pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, Calif).

Northern Blot Analysis

Northern blot analysis was performed as described previously.22 Briefly, total RNA was separated on a 1% agarose gel containing 2.2 M formaldehyde and transferred to a Hybond N membrane. The filter was washed once with 0.1% SDS in 0.2 sodium dodecyl sulfate (SDS) in 2× standard saline citrate (SSC) at 42°C for 24 hours. After hybridization, the membrane was washed once with 0.1% sodium dodecyl sulfate (SDS) in 2× SSC at room temperature (RT), and then washed 3 times with 0.1% SDS in 0.2× SSC at 65°C. Autoradiography was performed on an imaging plate and analyzed with a FLA 2000 (Fuji Film, Tokyo, Japan).

Western Blot Analysis

Polyclonal rabbit antibody against a synthetic peptide corresponding to the C-terminal 15 amino acids of Vezf1, including a cysteine linker (CAIGIKKEKKPTSVF), was prepared (anti-Vezf1 Ab). Western blot analysis was performed as described previously.23 Briefly, protein extracted from MSS31 cells was separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 hour at room temperature with Tris–HCl–buffered saline (TBS) containing 1% bovine serum albumin (BSA), and then incubated for 1 hour at reverse transcription in TBS containing 0.05% Tween 20 (T-TBS), 1% BSA, and anti-Vezf1 Ab diluted 1/1000, anti-stathmin/OP18 Ab diluted 1/100, anti-His6 diluted 1/100, or anti-β-actin antibody diluted 1/5000. The filter was then washed 4 times with T-TBS and incubated for 1 hour with horseradish peroxidase-conjugated protein G (Bio-Rad, Hercules, Calif). After the filter had been washed 3 times with T-TBS, the blots were detected by an enhanced chemiluminescence method using an ECL Western blotting detection kit (Amersham). The results were visualized using a LAS-1000 (Fuji Film).

Oligodeoxynucleotide Treatment

Oligodeoxynucleotide (ODN) treatment was performed as described previously.24 Briefly, MSS31 cells were incubated for 6 hours with ODNs in Lipofectin containing Opti-MEM at a fixed ratio of ODNs versus Lipofectin (1:3, eg, 1000 nM ODN per 3000 nM Lipofectin). Thereafter, ODN-containing medium was replaced with 10% FCS/αMEM. The sequences of Vezf1 ODNs used in this study were as follows: AS-ODN, 5’-TGACATAGTCCAGACGACA-3’, which is complementary to the mRNA region in Vezf1; sense (S-ODN), 5’-TGTGCTGGGAAGTATGACGTC-3’; mismatched (MIS-ODN), 5’-TGAGATAGACCCACAGTCA-3’, comprising AS-ODN with 4 base pairs substituted; and scrambled (SCR-ODN), 5’-CCAATGTGACAACTACAGC-3’, containing all the base pairs of the antisense codons in random order. The sequences of stathmin/OP18 ODNs used in this study were 5’-TCTGCGCTTCCGGTTCTC-3’ as AS-ODN and 5’-AGAAGACCGGAAGGGCAGA-3’ as S-ODN. No AS-ODN was homologous to any genes among the reported DNA sequences present in the BLAST program.

Cell Proliferation

MSS31 cells were inoculated at a density of 2×10⁴ per well into 96-well plates and were incubated for 6 hours with or without ODN. Thereafter, the medium was changed to 10% FCS/αMEM, and the cells were incubated for the desired period. Then, we added 10 µL TetraColor ONE (Seikagaku, Tokyo, Japan) to each well, and quantified the proliferation by use of a multiple-plate reader at 492 nm (Tosoh, Tokyo, Japan).

Cell Migration

After a 6-hour treatment with ODNs, cells were harvested with trypsin–EDTA, resuspended in 10% FCS/αMEM in a final volume of 100 µL, loaded (5×10⁴ cells per well) into a upper chamber of a Transwell Polycarbonate Membrane (pore size: 8 µm; Costar, Cambridge, Mass) containing 600 µL of 10% FCS/αMEM in the lower chamber, and incubated at 37°C for 3 hours. Cells on the lower surface were stained with Diff Quick kit (International Reagents, Kobe, Japan) and counted.

Network Formation

After a 6-hour treatment with ODNs, cells were harvested with trypsin–EDTA, resuspended in 10% FCS/αMEM in a final volume of 300 µL, replated (2×10⁵ cells per well) onto 24-well plates coated with Matrigel, and incubated at 37°C for 4 hours. Thereafter, cells were observed by phase-contrast microscopy. The lengths of network structures were quantified with microcomputer-assisted NIH Image software (National Institute of Health, Bethesda, Md) as described.24

Matrigel Implantation Analysis

Matrigel implantation analysis was performed as described previously.24 Briefly, male C57BL/6 mice at 4 weeks of age were used. We injected 300 µL growth factor-reduced Matrigel containing VEGF (100 ng/mL) plus heparin (32 U/mL) in liquid form at 4°C into the abdominal subcutaneous tissue at the mid peritoneal area of each mouse. In some experiments, 50 µmolar/L AS-ODN or S-ODN was mixed with the Matrigel.24 On day 6 after injection, the mice were euthanized and gels recovered. Animal study was reviewed and approved by the Committee for humane animal study in our Institute. For the quantification of angiogenesis, the Matrigel were weighed and homogenized in distilled water (10 µL/mg wet weight). The hemoglobin concentration in the supernatant after centrifugation at
5000g for 5 minutes was determined by use of a hemoglobin B-test kit (Wako, Osaka, Japan).

Immunohistochemical Analysis

Immunohistochemistry was performed as described previously.\(^{23}\) Anti-Vezf1 Ab, anti-von Willebrand factor Ab, and nonimmune rabbit immunoglobulin (Ig)-G were used as primary antibody, and were diluted 200-fold with PBS containing 1% BSA. The secondary antibody was biotin-conjugated antirabbit antibody diluted 200-fold with the same diluent. Coupling of streptavidin to biotin from an ABC kit (Vector Laboratories, Burlingame, Calif) was performed for 45 minutes at RT. Immunocomplexes were visualized by means of 3,3’-diaminobenzidine tablet sets (Sigma).

**cDNA Microarray Analysis**

After a 6-hour treatment with ODNs, the cells were incubated for 24 hours in 10% FCS/oMEM. Total RNA was isolated from Vezf1 AS and S-ODN-treated cells, and poly(A)\(^+\) RNA was purified by using an Oligotex-dt30 mRNA purification kit (Takara Shuzo, Shiga, Japan). The poly(A)\(^+\) RNA samples were analyzed using a mouse UniGEM V cDNA microarray containing 8735 genes (St. Louis, Mo). Cy3 and Cy5 were used for Vezf1 S-ODN and Vezf1 AS-ODN-treated cells, respectively. Genes with a Cy5 signal/Cy3 signal ratio >2.0 or <0.5 were considered to have changed in activity.

**Transient Transfection of Vezf1 Expression Vector**

The EcoRI fragment of the Vezf1 cDNA from pCR2.1-TOPO/Vezf1 was subcloned into the expression vector pcDNA4 (Invitrogen). MSS31 cells were transiently transfected with 10\(^{4}\) g of pcDNA4/Vezf1 or pcDNA4 vector mixed with Lipofectin. Transfected MSS31 cells were cultured in 10% FCS/oMEM. After 48 to 72 hours, total RNA and protein were extracted from cells for Northern and Western blot analyses.

**Cell Cycle Analysis**

After a 6-hour treatment with ODNs, cells were incubated in 10% FCS/oMEM for indicated periods. Thereafter, cells were harvested, fixed in ethanol (70%), suspended in PBS (500 \(\mu\)L), and incubated with RNase A (0.5 \(\mu\)g/\(\mu\)L). Nuclei of the cells were subsequently stained with propidium iodide (0.05 \(\mu\)g/\(\mu\)L) on ice for 1 hour. Stained nuclei of 10 000 cells were measured with a FACSScan flow cytometer (Becton Dickinson, San Jose, Calif), and the proportions of cells in the G0/G1, S, and G2/M phases of the cell cycle were analyzed by using the Modfit LT Software (Verity Software House, Topsham, Min).

**Trypan Blue Exclusion Assay**

Cells were incubated for 5 minutes in a solution of 0.2% trypan blue in PBS. More than 100 cells were counted in each field, and the percentage of nonviable cells was calculated.

**DNA Fragmentation Assay**

DNA was extracted from each group by using an Easy DNA kit (Invitrogen), and then the DNA (5 \(\mu\)g) was electrophoresed on a 2% agarose gel and stained with ethidium bromide.

**Results**

We examined whether Vezf1 was expressed in ECs at the site of angiogenesis in vivo. Immunostaining of the Matrigel with anti-Vezf1 Ab revealed positive staining of Vezf1 protein in ECs of neovessels, whereas nonimmune rabbit IgG gave a negative reaction (Figure 1A and 1C). Immunostaining with anti-von Willebrand factor Ab confirmed that almost all the cells in the Matrigel were ECs (Figure 1B).

The fact that Vezf1 was expressed in ECs at the site of angiogenesis promoted us to characterize whether this trans-
shown in Figure 2A, Vezf1 AS-ODN inhibited the proliferation of MSS31 cells, whereas control ODNs exhibited no effect. Vezf1 AS-ODN inhibited the proliferation in a dose-dependent manner over the range of 1 to 1000 nM (data not shown). Likewise, Vezf1 AS-ODN inhibited the migration (Figure 2B) or network formation (Figure 2C) by MSS31 cells, whereas control ODNs exhibited no effects.

We used Matrigel implantation analysis to show the requirement of Vezf1 in angiogenesis in vivo. Application of ODNs to this analysis was established and reported in the previous study. Matrigel containing VEGF and either Vezf1 AS or S-ODN was inoculated into mouse subcutaneous tissue. Invasion of new vessels into the gel was enhanced by VEGF (Figure 3A versus 3B). Vezf1 AS-ODN but not Vezf1 S-ODN apparently inhibited this process (Figure 3C versus 3D). Quantitative analysis confirmed the decrease in the levels of stathmin/OP18 mRNA and protein in Vezf1 AS-ODN–treated cells (Figure 4A and 4B). Conversely, transient transfections of Vezf1 gene augmented the expression of stathmin/OP18 mRNA and protein levels (Figure 4C and 4D). These results indicate that the expression of stathmin/OP18 is under the control of Vezf1 in ECs.

The question was whether Vezf1 and stathmin/OP18 were functionally related in regulation of angiogenesis. To test this, we downregulated the expression of stathmin/OP18. Stathmin/OP18 AS-ODN effectively and selectively inhibited the level of stathmin/OP18 mRNA and protein (Figure 3C and 3D). We then used these ODNs to show the requirement of stathmin/OP18 in angiogenesis. Stathmin/OP18 AS-ODN inhibited proliferation, migration, and network formation by MSS31 cells (Figure 5A through 5C).

Although stathmin/OP18 AS-ODN reproduced the inhibitory effects on angiogenesis-related properties of ECs, it might not directly prove the relationship between Vezf1 and stathmin/OP18. The characteristic feature of stathmin/OP18 and metallothionein 1 genes less than one-half of basal levels (Table I, available online at http://atvb.ahajournals.org). Because stathmin/OP18 was reported to be involved in cell division and cell migration, we focused on stathmin/OP18 in the present study. Northern and Western blot analyses confirmed the decrease in the levels of stathmin/OP18 mRNA and protein in Vezf1 AS-ODN–treated cells (Figure 4A and 4B). Conversely, transient transfections of Vezf1 gene augmented the expression of stathmin/OP18 mRNA and protein levels (Figure 4C and 4D). These results indicate that the expression of stathmin/OP18 is under the control of Vezf1 in ECs.
inhibition is mitotic abnormalities during the cell division and apoptosis.29 We therefore tested whether Vezf1 AS-ODN could cause the same abnormalities as stathmin/OP18 AS-ODN did. Both Vezf1 and stathmin/OP18 AS-ODNs decreased G2/M-phase population of MSS31 cells at a 12-hour time point (Figure 6A). Moreover, Vezf1 and stathmin/OP18 AS-ODNs increased cell death at a 2-hour time point (Figure 6B). This cell death appeared to be apoptosis, since typical DNA ladders were observed in Vezf1 and stathmin/OP18 AS-ODNs treated MSS31 cells (Figure 6C).

**Discussion**

Vezf1 is a transcription factor expressed in ECs during vascular formation in mouse embryo.19 Here, we showed that Vezf1 was expressed in ECs at the site of angiogenesis. Moreover, the specific elimination of Vezf1 expression abrogated proliferation, migration and network formation by cultured ECs as well as angiogenesis in vivo. Thus, Vezf1 is involved in the regulation of angiogenesis.

As Vezf1 is a transcription factor, Vezf1 should regulate angiogenesis by modulating downstream target genes. To search the target genes of Vezf1 in ECs, we performed cDNA microarray analysis. Although endothelin-1 is reported to be the candidate target of Vezf1 in ECs,30 the mouse cDNA microarray system that we used did not contain endothelin-1 gene, and thus we could not find whether the expression of endothelin-1 was under the control of Vezf1 in our system. Instead, stathmin/OP18 gene and metallothionein 1 gene were downregulated to less than one-half of basal levels. Here, we focused on stathmin/OP18. Stathmin/OP18 AS-ODN reproduced the inhibitory effects of Vezf1 AS-ODN on the properties of ECs related to angiogenesis. More importantly, Vezf1 AS-ODN exhibited the abnormality in cell cycle progression and apoptosis, which was characteristic to stathmin/OP18 inhibition.29 Thus, our results suggest that Vezf1 and stathin/OP18 are functionally related in the regulation of angiogenesis.

Apoptosis is thought to be the result of cell cycle arrest achieved by microtubule stabilization. Cell cycle arrest was observed after 12-hour incubation. At that time, we could not show the DNA ladder formation even in the concentration of 1000 nM of Vezf1 AS-ODN, whereas migration and network
formation were inhibited within this incubation period. The DNA ladder formation was observed from the 24-hour point. Thus, apoptosis of ECs is not a specific event for the inhibition of angiogenesis.

Stathmin/OP18 is a 19-kDa cytoplasmic protein, whose function is related to microtubule turnover. Microtubules, polymers composed of self-assembling α- and β-tubulin heterodimers, display kinetic polarity in that the rate of polymerization is different at the 2 ends; the faster growing end is the plus end (β-tubulins exposed), and the slower growing end is the minus end (α-tubulins exposed). The ends of microtubules switch stochastically between phases of slow growth and rapid shrinkage, and the transition from growth to shrinkage is called tubulin catastrophe. Thus, stathmin/OP18 increases microtubule turnover and contributes to rapid reorganization of the microtubule cytoskeleton.

Microtubules are essential for a wide variety of cellular functions including mitosis, cell shape, and cell motility. Stathmin/OP18 should have influences on these phenomena. Stathmin/OP18 is involved in the migration of various cell types including olfactory epithelial cells and germ cells. We observed that inhibition of either stathmin/OP18 or Vezf1 inhibited the migration of ECs. More importantly, the inhibition of stathmin/OP18 expression leads to the decrease of cell division and subsequent apoptosis. Here, we observed that inhibition of stathmin/OP18 as well as that of Vezf1 suppressed cell cycle progression of ECs and induced apoptosis. Taken together, our results suggest that Vezf1 and stathmin/OP18 are functionally related.

Tubulin is one of the major targets of drug development. Consequently, tubulin inhibitors have attracted great attention as anticancer agents for chemotherapeutic use. Moreover, a close correlation between tubulin turnover and angiogenesis is disclosed from the anti-angiogenic effect of such anticancer drugs. For example, a noncytotoxic low dose of paclitaxel effectively inhibited angiogenesis. Paclitaxel promotes polymerization of tubulin dimers to form and to stabilize microtubules. Because stathmin/OP18 inhibition also stabilizes microtubules, the way of paclitaxel to inhibit angiogenesis should be comparable to that of stathmin/OP18 inhibition.

In summary, our present results indicate for the first time to our knowledge that Vezf1 plays an important role in angiogenesis, at least in part, through the expression of stathmin/OP18. The present findings should guide us to an innovative avenue of angiogenesis regulation regarding tubulin turnover in ECs.

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References


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## Supplementary Table.  Expression gene profiling in Vezf1 AS-ODN treated MSS31 cells

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Supplementary Figure:

MSS31 cells were treated with VEZF1 ODNs (1,000 nM) for 6 h. Thereafter, the cells were cultured in 10% FCS/αMEM. Total RNAs (12 h after the treatment) and proteins (18 h after the treatment) were isolated, and Northern (A) and Western (B) blot analyses were performed. MSS31 cells were treated with stathmin/OP18 ODNs (1,000 nM). Northern (C) and Western (D) blot analyses were performed. As a control, 28S RNA from the ethidium bromide-stained gel or β-actin was shown.