Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 316 (2004) 52–58
BBRC
www.elsevier.com/locate/ybbrc

Transcriptional regulation of human angiopoietin-2 by transcription factor Ets-1

Yasuhiro Hasegawa,[a,b] Mayumi Abe,[a] Tohru Yamazaki,[a] Osamu Niizeki,[a] Kenichi Shiiba,[b] Iwao Sasaki,[b] and Yasufumi Sato[a,*]

[a] *Department of Vascular Biology, Institute of Development, Aging, and Cancer, Tohoku University, 4-1, Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan*
[b] *Division of Biological Regulation and Oncology, Department of Surgery, Tohoku University Graduate School of Medicine, 1-1, Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan*

Received 19 December 2003

Abstract

Angiopoietin-2 (Ang-2) plays an important role in destabilizing vessels for angiogenesis, however its transcriptional regulation has not been determined. Here we isolated about 3.2 kb of the 5′ upstream of the human Ang-2 gene and further characterized the transcriptional regulation of Ang-2. We found 10 Ets binding sites (EBSs) in this region. Ets-1 and Ets-2 but not Erg-1 augmented its promoter activity. Sequential deletion of EBSs and mutation analysis revealed that EBS8 located at −416/−406 from the translation initiation site was critical for Ets-1- or Ets-2-stimulated promoter activity. VEGF induced the expression of Ets-1 and Ang-2 in human umbilical vein endothelial cells (HUVECs). Electrophoretic mobility shift and chromatin immunoprecipitation assays showed that Ets-1 bound to EBS8 in HUVECs. Moreover, synthetic double-strand oligonucleotides containing corresponding EBS8 abrogated the induction of Ang-2 by VEGF in HUVECs. These results indicate that Ets-1 and the corresponding EBS are critical for the induction of Ang-2.

Keywords: Angiopoietin-2; Promoter; Transcriptional factor; Ets-1; VEGF; Endothelial cell

Angiogenesis is a process by which new blood vessels are formed from pre-existing ones. Physiological angiogenesis in the adult is observed only in restricted sites such as the endometrium and ovarian follicle, and it is normally transient. However, persistent angiogenesis plays a crucial role in pathologic states including tumors [1]. Angiogenesis is normally achieved by the following sequential steps: detachment of mural pericytes for vascular destabilization, extracellular matrix degradation, migration of endothelial cells (ECs), proliferation of ECs, tube formation by ECs, and reattachment of pericytes for vascular stabilization [2].

Many factors are reported to be involved in angiogenesis. Among them, endothelium-tropic factors including vascular endothelial growth factor (VEGF) family members and angiopoietins play principal roles. Angiopoietin-1 (Ang-1) is an agonistic ligand of the TIE-2 receptor and stabilizes the vessels by promoting the adhesive interactions between ECs and mural cells [3,4]. In contrast, angiopoietin-2 (Ang-2) induces detachment of mural cells and destabilizes the vessels [5]. Thus far, the mechanism by which Ang-2 promotes the detachment of the pericytes remains elusive. Ang-2 can transduce a very weak signal through the shared receptor TIE-2 [6]. However, various data indicate that Ang-2 may be a natural antagonist of Ang-1 and that pericyte detachment induced by Ang-2 may be the result of competitive inhibition of Ang-1 binding to TIE-2. Destabilization by Ang-2 in the absence of VEGF is proposed to result in vessel regression [7], whereas such destabilization in the presence of VEGF facilitates the angiogenic response [8]. In spite of these findings, the transcriptional regulation of Ang-2 has remained largely unknown.

* Corresponding author. Fax: +81-22-717-8533.
E-mail address: y-sato@idac.tohoku.ac.jp (Y. Sato).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.02.019

The Ets family proteins are transcription factors that bind to the regulatory region of target genes via the Ets binding motif, GGAA/T [9]. Ets-1 is a prototype of this family. Ets-1 is expressed in ECs during angiogenesis or re-endothelialization after denuding injury [10–12]. Ets-1 is induced in ECs by representative angiogenic factors such as VEGF and basic fibroblast growth factor [11,13], and VEGF-stimulated induction of Ets-1 is mediated through VEGF type-2 receptor and its downstream extracellular signal-regulated protein kinase1/2 [14,15]. Ets-1 promotes angiogenesis by inducing urokinase-type plasminogen activator, matrix metalloproteinase (MMP)-1, MMP-3, MMP-9, and integrin β3 in ECs as target genes [11,16], and the transfection with a truncated Ets-1 gene having dominant-negative activity inhibits angiogenesis [17].

Earlier we transiently overexpressed Ets-1 in human umbilical vein endothelial cells (HUVECs) and characterized the uncovered function of Ets-1 [18]. A comprehensive search by cDNA microarray analysis revealed that Ets-1 augmented various angiogenesis-related genes including Ang-2 in HUVECs [19]. Here, we characterized the promoter region of the human Ang-2 gene and identified a cluster of Ets binding sites (EBSs). We further determined the critical EBS responsible for the transactivation of the human Ang-2 gene.

Materials and methods

Materials. Recombinant human VEGF ($VEGF_{165}$) was purchased from Sigma (St. Louis, MO); rabbit polyclonal anti-human Ets-1 antibody (Ab), anti-human Ets-2 Ab, anti-human Erg-1 Ab, and rabbit normal IgG were from Santa Cruz Biotechnology (Santa Cruz, CA); T4 polynucleotide kinase was from Takara (Ohtsu, Japan); poly(dI–dC)·poly(dI–dC) and Sephadex G-50 (DNA grade) were from Iwaki (Tokyo, Japan); Opti-MEM and Lipofectin were from Gibco-BRL (Rockville, MD); PGL3 vector, pRL-TK vector, and Dual-Luciferase reporter assay system were from Promega (Madison, WI); and [α-^{32}P]dCTP and [γ-^{32}P]ATP were from Amersham (Buckinghamshire, UK).

Cells. HUVECs were obtained from Kurabo (Osaka, Japan) and cultured on type-I collagen-coated dishes (IWAKI) in endothelial basal medium (EBM) containing endothelial cell growth supplement (Clonetics, Walkersville, MD) and 10% fetal calf serum (FCS) (JRH Biosciences, San Antonio, TX). COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) containing 10% FCS, 4 mM glutamate, 100 μg/ml kanamycin, and 2.2 mg/ml $NaHCO_3$.

Isolation and characterization of genomic clones. BAC Human Filter (Incyte Genomics, Palo Alto, CA) was hybridized with radiolabeled cDNA of human Ang-2. BAC-positive clone was obtained from Incyte Genomics and analyzed by sequencing with appropriate gene-specific primers. The nucleotide sequences were determined by using a DNA sequencing kit (Applied Biosystems, Foster City, CA) and an ABI Prism 310 DNA sequencer (Applied Biosystems).

Plasmid constructions. A 3.20-kb of genomic sequence upstream to the translation initiation site of the human Ang-2 promoter sequence was amplified by polymerase chain reaction (PCR) from the BAC-positive clone by using pyrobest DNA polymerase (Takara) and the oligonucleotide primer pairs of 5′-CTAGCTAGCTGCTTTGCA CCGCATT-3′ and 5′-CCCAAGCTTAAC TTAACTTGAGGGCA AACACAC-3′. This fragment was digested with *Nhe*I and *Hin*dIII and subcloned into the *Nhe*I and *Hin*dIII sites of the pGL3 basic vector, which contains the coding region for firefly luciferase. Deletion constructs to remove successive EBSs were made by PCR amplification using the oligonucleotides 5′-CTAGCTAGCTTGTGCACCCGGA AGCATCA-3′ and 5′-CCCAAGCTTAACTTAACTTGAGGGCAA ACACAC-3′ (deletion of EBS1), 5′-CTAGCTAGCCTGGGCAAC AAGAGTGAGA-3′ and 5′-CCCAAGCTTAACTTA ACTTGAGG GCAAACACAC-3′ (deletion of EBS2), 5′-CTAGCTAGCAAGGCT GTCTTTCGGTTAG-3′ and 5′-CCCAAGCTTAACTAACTTGAG GGCAAACACAC-3′ (deletion of EBS3), 5′-CTAGCTAGCCATGG TGAAACCCTGTCTC-3′ and 5′-CCCAAGCTTAACTTAACTTGA GGGCAAACACAC-3′ (deletion of EBS4), 5′-CTAGCTAGCACG TTTACAGGAGCCAAAC-3′ and 5′-CCCAAGCTTAACTTGAGG GCAAACACAC-3′ (deletion of EBS5), 5′-CTAGCTAGCTATTTT GCCAGCTTAGCAC-3′ and 5′-CCCAAGCTTAACTTAACTTGA GGGCAACACAC-3′ (deletion of EBS6), 5′-CTAGCTAGCCCCCT ACAGGAAGATAACGG-3′ and 5′-CCCAAGCTTAACTTA ACT TGAGGGCAAACACAC-3′ (deletion of EBS7), 5′-CTAGCTAGC CCACTACACATGTCTGGCTG-3′ and 5′-CCCAAGCTTAACTT AACTTGAGGGCAAACACAC-3′ (deletion of EBS8), and 5′-CT AGCTAGCACTGACACTGTAGGATCTGG-3′ and 5′-CCCAAGC TTAACTTGAGGGCAAACACAC-3′ (deletion of EBS9). To make the construct of mutated EBS8, we performed PCR amplification using oligonucleotides 5′-CTAGCTAGCCCCCTACACCAAGATAACG G-3′ and 5′-CCCAAGCTTAACTTAACTTGAGGGCAAACACAC-3′. These PCR products were subcloned into the *Nhe*I and *Hin*dIII sites of the pGL3 basic vectors. All constructs and a mutated region were sequenced to check for any spurious changes.

Human Ets-1, Ets-2, and ets-related gene (Erg)-1 cDNAs were kindly provided by Dr. Dennis K. Watson (Medical University of South Carolina). The entire coding regions of Ets-1 and Ets-2 were amplified by PCR using the primer oligonucleotides 5′-CCCAA GCTTCATGAAGGCGGCCGTCGAT-3′ and 5′-CCGGATATCTG CTCGTCGGCATCTGGCTT-3′ (Ets-1), 5′-CCCAAGCTTGATGA ATGATTTC-3′ and 5′-CCGGATATCTGGTCCTCCGTGTC-3′ (Ets-2) having *Hin*dIII and *Eco*RV sites. The amplified products were digested with *Hin*dIII and *Eco*RV, and subcloned into pcDNA3.1/Myc-HisC (Invitrogen, Carlsbad, CA). For the entire coding region of Erg-1, primer oligonucleotides 5′-TTGGCGGCCGCTATGGTGGG CAGCCCAGA-3′ and 5′-TCCCCTCGAGCTTAGTAGTAAGTGC CCA-3′ having *Not*I and *Xho*I sites were used. The amplified product was digested with *Not*I and *Xho*I and subcloned into pcDNA3.1/Myc-HisC.

Luciferase assay. COS7 cells (2×10^4) were seeded into 24-well tissue culture dishes and incubated for 16 h. Thereafter, the cells were transfected by use of Lipofectin for 3 h with 0.4 μg Ang-2 reporter plasmid, 0.04 μg pRL-TK plasmid, which contains a herpes simplex virus thymidine kinase promoter upstream of the *Renilla* luciferase gene, and 0.2 μg of pcDNA3.1/Myc-HisC empty vector or vector for expression of Ets-1, Ets-2, or Erg-1. After a 21-h incubation in DMEM containing 10% FCS, the cells were washed with PBS and lysed with passive lysis buffer. Firefly luciferase and *Renilla* luciferase activities were measured sequentially by using a Dual-Luciferase Reporter assay system and a Luminescencer JNR (Atto, Tokyo, Japan). After the firefly luciferase signal (FLS) and the *Renilla* luciferase signal (RLS) had been measured, the relative luciferase activity was calculated as FLS/RLS.

Western blot analysis. Western blot analysis was carried out as described previously [11]. Briefly, 24 h after the transfection, protein was extracted from COS7 cells, separated by SDS–PAGE, and transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature with Tris–HCl-buffered saline (TBS) containing 1% bovine serum albumin (BSA) and then incubated for 1 h at RT in TBS containing 0.05% Tween 20 (T-TBS), 1% BSA, and either anti-Ets-1 Ab diluted 1/200, anti-Ets-2 Ab diluted 1/200, anti-Erg-1 Ab

diluted 1/200, or anti-β-actin antibody diluted 1/5000. The filter was then washed four times with T-TBS and incubated for 1 h with horseradish peroxidase-conjugated protein G (Bio-Rad, Hercules, CA). After the filter had been washed three 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 by using a LAS-1000 (Fuji, Tokyo, Japan).

Electrophoretic mobility shift assay (EMSA). For COS7 cells, cells in 10-cm plastic dishes were transfected by use of Lipofectin for 3 h with or without 4 μg of Ets-1-expressing vector and then incubated for 24 h in DMEM containing 10% FCS. For HUVECs, cells were preincubated for 16 h in M199 containing 5% FCS and then stimulated with 1 nM VEGF for 12 h. Thereafter, whole cell extracts (WCEs) were prepared by the freezing–thawing method. Synthetic oligonucleotides (normal EBS8, 5′-CCCCTACAGGAAGATAACGG-3′; and mutant EBS8, 5′-CCCCTACACCAAGATAACGG-3′) were labeled by use of T4 polynucleotide kinase and [γ-^{32}P]dATP. Each DNA was purified by Sephadex G-50 column chromatography and diluted to 10,000 cpm/μl. Two microliters of DNA was incubated in a final volume of 20 μl with 1.5 μg poly(dI–dC)·poly(dI–dC), 10 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT, and 5 μg WCE for 30 min at room temperature. In some experiments, 3 μg of WCE was incubated with 5 μg anti-Ets-1 antibody or the same amount of non-immune rabbit IgG at 4 °C for 1 h prior to the addition of ^{32}P-labeled DNA. For competition experiments, a 100-fold amount of unlabeled oligonucleotide was added to the reaction. The mixture was separated on a native 4% polyacrylamide gel in 1× TAE for 90 min. Autoradiography was carried out with an imaging plate and the bands were analyzed with a BAS2000 Image Analyzer (Fuji).

Chromatin immunoprecipitation assay. The chromatin immunoprecipitation (ChIP) assay was performed by using a Chromatin Immunoprecipitation Assay Kit (Upstate Biotech, Lake Placid, NY). Approximately 1×10^7 of HUVECs were stimulated with 1 nM VEGF for 12 h. Thereafter, formaldehyde was added to the culture media to a final concentration of 1% and the cells were then incubated at 37 °C for 10 min. Next, they were washed twice with ice-cold PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg/ml aprotinin, scraped into the same buffer, and then pelleted. The pellets were resuspended in 0.1 ml SDS lysis buffer and incubated for 10 min on ice. The cell lysates were sonicated six times, 10 s each time, and centrifuged. Supernatants were diluted 10-fold by addition of ChIP dilution buffer and immunoprecipitated with 2 μg of anti-Ets-1 antibody or non-immune rabbit IgG for 16 h at 4 °C. Sixty microliters of salmon sperm DNA/protein-A–agarose–50% slurry was added to the tubes, which were then rotated for 1 h at 4 °C. After a gentle centrifugation, the pellets were collected and washed sequentially with low-salt buffer, high-salt buffer, LiCl buffer, and TE (5 min for each). Immunoprecipitates were resuspended in 100 μl elution buffer (1% SDS, 0.1 M $NaHCO_3$), mixed with 4 μl of 5 M NaCl, and placed at 65 °C for 6 h. Thereafter, DNA was extracted by phenol/chloroform and ethanol precipitation, resuspended in 50 μl of water, and amplified by PCR. The primer set was as follows: sense, 5′-TATTTTGCCAGCTTAGCA C-3′; and antisense, 5′-ATCCTACAGTGTCAGTATCC-3′. To show the input of sonicated chromatin used in the ChIP assays, we applied 0.5% of sonicated chromatin before immunoprecipitation.

Application of a transcription factor decoy and Northern blotting. Cells were treated with a transcription factor decoy as described previously [20]. Briefly, by use of Lipofectin, subconfluent HUVECs were transfected with synthetic double-strand oligonucleotides (1 μM) at 37 °C for 3 h and then incubated in M199 containing 5% FCS for 12 h. After the incubation, the cells were stimulated with 1 nM VEGF for 12 h; and total RNA was then extracted by the AGPC method, fractionated on a 1% agarose gel containing 2.2 M formaldehyde, and transferred onto a nylon filter (Hybond N+, Amersham). The filter was hybridized with ^{32}P-labeled probe in hybridization solution for 24 h at 42 °C. Thereafter, the filter was washed in 2× SSC and 0.1% SDS at 42 °C and then in 0.2× SSC and 0.1% SDS at 65 °C. Autoradiography was carried out and analyzed with a BAS2000 Image Analyzer (Fuji).

Results

Ets-1 activates the Ang-2 promoter activity

The region approximately 3.2-kb upstream from the translation initiation site of the human Ang2 gene was isolated. This region was placed in front of a luciferase reporter gene and the promoter activity was analyzed. Hypoxia was reported to increase the expression of Ang-2 [21]. However, we could not find any hypoxia

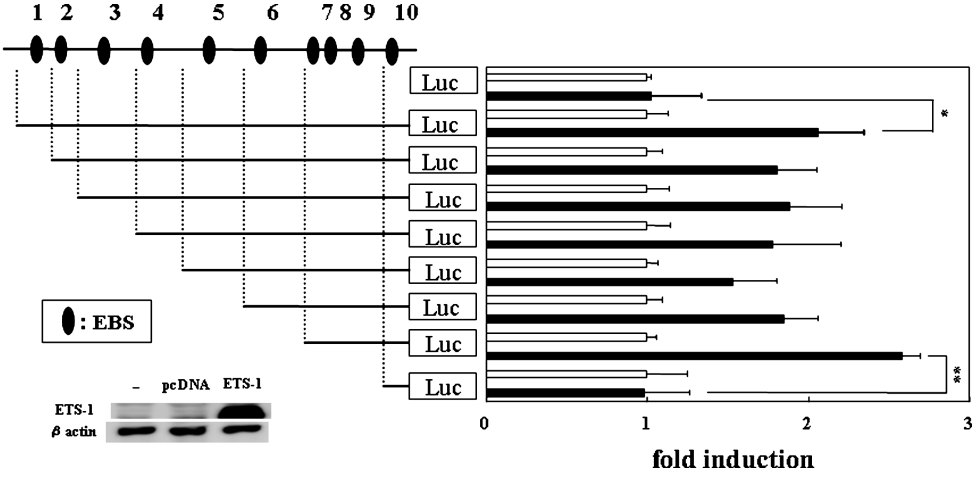

Fig. 1. Transcriptional activation of human Ang-2 promoter by Ets-1. In a 3.2-kbp upstream region starting from the start codon of the human Ang-2 promoter, 10 EBSs were found and named as shown at the top of the figure. COS7 cells were transiently co-transfected with each promoter construct and pcDNA3.1 empty vector (open bars) or Ets-1-expressing vector (solid bars). Western blotting showed that the transfection with Ets-1-expressing vector induced the synthesis of Ets-1 protein. Luciferase activity was expressed as fold induction for each construct. Values are means of triplicate samples. Error bars indicate standard deviations (SDs). $*P < 0.05$, $**P < 0.005$.

responsive elements in this region and hypoxia did not increase the promoter activity (data not shown). Instead, we found 10 EBSs. As shown in Fig. 1, co-transfection of COS7 cells with Ets-1-expressing vector and the Ang-2 full promoter construct significantly increased the relative luciferase activity compared with that obtained with the empty vector. Sequential deletion of these EBSs showed that the relative luciferase activity was significantly decreased when the cluster of EBS7, EBS8, and EBS9 was deleted. The nucleotide sequence around this region is shown in Fig. 2A. Additional deletion constructs in this region showed that the relative luciferase activity was aborted when the EBS8 (−416/−406) was deleted (Fig. 2B). Furthermore, mutation of EBS8 (GGAA to CCAA) significantly decreased the responsiveness of the promoter fragment to Ets-1 (Fig. 2C). These results thus indicate that EBS8 is critical for the promoter activity of human Ang-2.

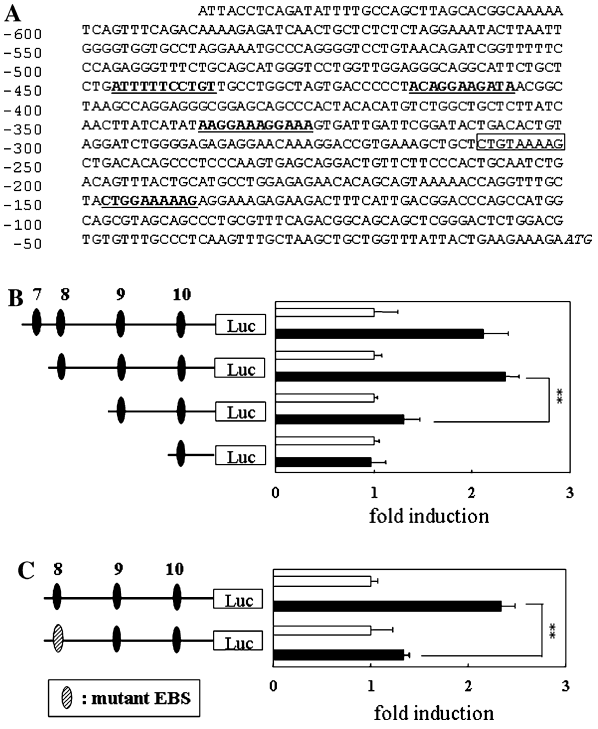

Fig. 2. EBS responsible for the transactivation of human Ang-2. (A) Nucleotide sequence of human Ang-2 promoter. Bolded and underlined sequences are EBSs (EBS 7–10). Box indicates the TATA box. (B) Effect of progressive deletion of EBSs on Ang-2 promoter activity by Ets-1. COS7 cells were transiently transfected with each promoter construct and pcDNA3.1 empty vector (open bars) or Ets-1-expressing vector (solid bars), and luciferase activity was expressed as fold induction for each construct. (C) Effect of EBS8 mutation on Ang-2 promoter activity by Ets-1. COS7 cells were transiently transfected with each promoter construct along with pcDNA3.1 empty vector (open bars) or Ets-1-expressing vector (solid bars), and luciferase activity was expressed as fold induction for each construct. Values are means of triplicate samples. Error bars indicate SDs. $^{**}P < 0.005$.

We examined other Ets family transcription factors. Co-transfection of COS7 cells with the Ets-2-expressing vector significantly increased the relative luciferase activity compared with the activity obtained with the empty vector, and the deletion of EBS8 decreased the relative luciferase activity (Figs. 3A–C). In contrast, co-transfection with the Erg-1-expressing vector did not increase the Ang-2 promoter activity at all (Fig. 3D).

Ets-1 binds to the EBS8 element

We performed EMSA analysis to show the binding of Ets-1 to the EBS8. First, WCEs from COS7 cells transfected with the Ets-1-expressing vector were applied to EMSA. A DNA–protein complex was detected by using the labeled normal EBS8 (Fig. 4A, lane 3). An excess amount of unlabeled normal EBS8 competed with the labeled one for the complex formation (Fig. 4A, lane 4), whereas an excess amount of unlabeled mutant EBS8 did not (Fig. 4A, lane 5).

We previously demonstrated that VEGF induced Ets-1 as well as Ang-2 in ECs [11,13,22]. We therefore stimulated HUVECs with VEGF and obtained WCEs from HUVECs. As shown in Fig. 4B, the density of the DNA–protein complex band was enhanced by the stimulation with VEGF. This increased DNA–protein complex was competed by an excess of unlabeled normal EBS8 but not by one of mutant EBS8 (Fig. 4C, lane 4 vs. 5). Moreover, the band density apparently decreased when anti-Ets-1 antibody, but not non-immune IgG, was added to WCSs with the labeled EBS8 probe (Fig. 4C, lane 2 vs. 3). These results indicate that the band observed in VEGF-stimulated HUVECs represents the complex of Ets-1 and the labeled EBS8 probe.

EBS8 is important for transcriptional activity of Ang-2 in HUVECs

We performed ChIP analysis to confirm the binding of Ets-1 to this region of the Ang-2 gene. PCR amplification of the DNA fragments isolated from VEGF-stimulated HUVECs, with the primers that allowed detection of EBS8, showed that Ets-1 was present in this region (Fig. 5A).

To further verify the requirement of EBS8 in VEGF-stimulated induction of Ang-2 in HUVECs, we applied double-stranded oligonucleotides of the normal EBS8 and the mutant EBS8 as transcription factor decoys. Northern blotting revealed that VEGF induced the expression of Ang-2 (Fig. 5B; lane 2); and the treatment with the normal EBS8 almost completely inhibited this induction of Ang2 (Fig. 5B; lane 3), whereas the treatment with the mutant EBS8 had little effect (Fig. 5B; lane 4). These results indicate that Ets-1 is involved at least in part in the VEGF-stimulated induction of Ang-2 in HUVECs.

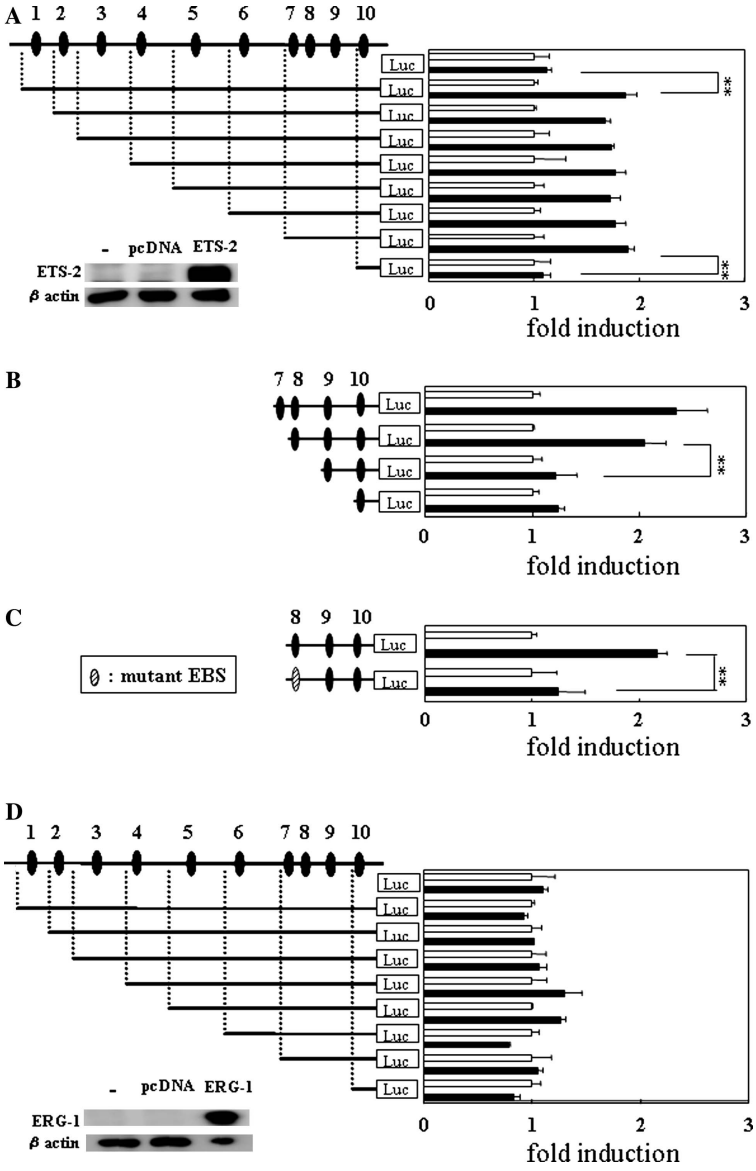

Fig. 3. Role of Ets-2 and Erg-1 in the transcriptional activation of human Ang-2 promoter. (A) Same as Fig. 1 except that the Ets-2-expressing vector (solid bars) was used in place of the Ets-1-expressing one. Western blotting showed that the transfection with Ets-2-expressing vector induced the synthesis of Ets-2 protein. (B,C) Same as Figs. 2B and C, respectively, except that the Ets-2-expressing vector replaced the Ets-1-expressing one. (D) COS7 cells were transiently co-transfected with each promoter construct. Each promoter construct along with pcDNA3.1 empty vector (open bars) or Erg-1-expressing vector (solid bars). Western blotting showed that the transfection with Erg-1-expressing vector induced the synthesis of Erg-1 protein. Luciferase activity was expressed as fold induction for each construct. Values are means of triplicate samples. Error bars indicate SDs. $^{**}P < 0.005$.

Discussion

The significance of Ang-2 has been shown in the embryonic or peri-natal vascular development [4,23]. Moreover, accumulating evidence suggests that Ang-2 also plays a crucial role in pathological angiogenesis including tumors. Ang-2 is up-regulated in ECs of tumor vessels [24]. Unlike normal blood vessels, tumor blood vessels are tortuous, leaky, and immature [25]. The abnormal architectures of tumor vessels may be the consequence of Ang-2 up-regulation, since the gene transfer of Ang-1 in tumor cells enhanced the coverage of vessels with pericytes and made vessel mature [26]. Importantly, the angiogenic response in advanced tumors requires the concomitant expression of Ang-2 and VEGF [6]. Whereas Ang-1 and Tie-2 expression levels were rather stable, the expression of Ang-2 in tumor vessels was closely correlated with the amount of VEGF in advanced tumors [27]. VEGF induces the expression of Ang-2 in ECs [21,22]. Thus, the up-regulation of Ang-2 by VEGF is thought to be a characteristic feature in advanced tumors.

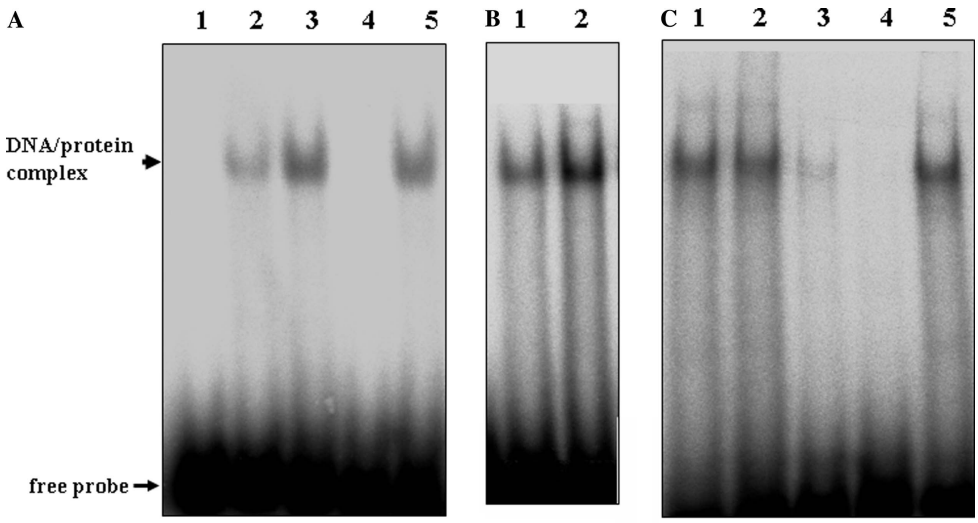

Fig. 4. Binding of Ets-1 to EBS8 of the human Ang-2 promoter. (A) WCEs isolated from COS7 cells were applied to EMSA. Lane 1, ^{32}P-labeled EBS8 probe alone; lane 2, WCE isolated from untransfected COS7 cells; lanes 3–5, WCE isolated from COS7 cells transfected with Ets-1-expressing vector: no additive (lane 3), 100-fold unlabeled normal EBS8 was added (lane 4), and 100-fold unlabeled mutant EBS8 was added (lane 5). (B) WCEs from HUVECs stimulated with or without VEGF (50 ng/ml) were used for the EMSA. Lane 1, unstimulated HUVECs; lane 2, VEGF stimulation. (C) WCEs from HUVECs stimulated with VEGF (50 ng/ml) were used for the EMSA. Lane 1, WCE alone; lane 2, WCE with nonimmune anti-rabbit IgG; lane 3, WCE with anti-Ets-1 antibody; lane 4, WCE with 100-fold unlabeled normal EBS8; and lane 5, WCE with 100-fold unlabeled mutant EBS8.

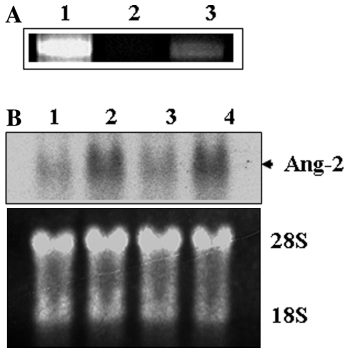

Fig. 5. Ets-1 bound to EBS8 is responsible for the induction of Ang-2 in HUVECs. (A) The ChIP assay was performed as described in Materials and methods. Lane 1, PCR amplification before immunoprecipitation; lane 2, PCR amplification after immunoprecipitation with nonimmune anti-rabbit IgG; and lane 3, PCR amplification after immunoprecipitation with anti-Ets-1 antibody. (B) HUVECs were treated with double-stranded oligonucleotides of normal EBS8 (normal decoy) or mutant EBS8 (mutant decoy). Thereafter, the cells were stimulated with VEGF (50 ng/ml). The expression of Ang-2 mRNA was determined by Northern blotting. Lane 1, unstimulated HUVECs; lane 2, VEGF stimulation; lane 3, normal decoy with VEGF stimulation and lane 4, mutant decoy with VEGF stimulation.

Here we demonstrated for the first time the importance of Ets family transcription factor in the induction of Ang-2. A question arises as to whether or not such a scenario operates in the in vivo situation. Several Ets family proteins have been studied in terms of the regulation of angiogenesis [28]. Here we showed that Ets-1 and Ets-2 exhibited similar effects on the promoter activity of Ang-2, whereas Erg-1 has no such effect. Ets-1 and Ets-2 are closely related, but Ets-1 is preferentially expressed in ECs of developing vessels [29]. Notably, Ets-1 is the only Ets family protein that is induced by VEGF stimulation and is up-regulated in vessels of various tumors [10,30–32]. Moreover, the expression of Ets-1 in tumor vessels is positively correlated with the amount of VEGF in tumors [31,33]. These series of observations strongly suggest that VEGF in tumors augments the expression of Ang-2 in ECs via the induction of Ets-1.

In conclusion, our present study demonstrates the essential EBS in the transactivation of the human Ang-2 gene. We further propose that Ets-1 induced by VEGF may represent one of the critical transcription factors in the regulation of Ang-2 gene expression in human ECs.

Acknowledgment

This work was supported by the Japan Society of the Promotion of Science Research for the Future (Grant No. 99L01304).

References

[1] J. Folkman, Angiogenesis in cancer, vascular, rheumatoid and other diseases? Nat. Med. 1 (1995) 27–31.
[2] D. Hanahan, Signaling vascular morphogenesis and maintenance, Science 277 (1997) 48–50.
[3] S. Davis, T.H. Aldrich, P.F. Jones, A. Acheson, D.L. Compton, V. Jain, T.E. Ryan, J. Bruno, C. Radziejewski, P.C. Maisonpierre, G.D. Yancopoulos, Isolation of angiopoietin-1, a ligand for the

TIE-2 receptor, by secretion-trap expression cloning, Cell 87 (1996) 1161–1169.
[4] C. Suri, P.F. Jones, S. Patan, S. Bartunkova, P.C. Maisonpierre, S. Davis, T.N. Sato, G.D. Yancopoulos, Requisite role of angiopoietin-1, a ligand for the TIE-2 receptor, during embryonic angiogenesis, Cell 87 (1996) 1171–1180.
[5] P.C. Maisonpierre, C. Suri, P.F. Jones, S. Bartunkova, S.J. Wiegand, C. Radziejewski, D. Compton, J. McClain, T.H. Aldrich, N. Papadopoulos, T.J. Daly, S. Davis, T.N. Sato, G.D. Yancopoulos, Angiopoietin-2, a natural antagonist for Tie-2 that disrupts in vivo angiogenesis, Science 277 (1997) 55–60.
[6] I. Kim, J.H. Kim, S.O. Moon, H.J. Kwak, N.G. Kim, G.Y. Koh, Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway, Oncogene 19 (2000) 4549–4552.
[7] J. Holash, P.C. Maisonpierre, D. Compton, P. Boland, C.R. Alexander, D. Zagzag, G.D. Yancopoulos, S.J. Wiegand, Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF, Science 284 (1999) 1994–1998.
[8] T. Asahara, D. Chen, T. Takahashi, K. Fujikawa, M. Kearney, M. Magner, G.D. Yancopoulos, J.M. Isner, Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization, Circ. Res. 83 (1998) 233–240.
[9] A.D Sharrocks, The ETS-domain transcription factor family, Nat. Rev. Mol. Cell. Biol. 2 (2001) 827–837.
[10] N. Wernert, M.B. Raes, P. Lassalle, M.P. Dehouck, B. Gosselin, B. Vandenbunder, D. Stehelin, C-ets1 proto-oncogene is a transcription factor expressed in endothelial cells during tumor vascularization and other forms of angiogenesis in humans, Am. J. Pathol. 140 (1992) 119–127.
[11] C. Iwasaka, K. Tanaka, M. Abe, Y. Sato, Ets-1 regulates angiogenesis by inducing the expression of urokinase-type plasminogen activator and matrix metalloproteinase-1 and the migration of vascular endothelial cells, J. Cell Physiol. 169 (1996) 522–531.
[12] K. Tanaka, N. Oda, C. Iwasaka, M. Abe, Y. Sato, Induction of Ets-1 in endothelial cells during reendothelialization after denuding injury, J. Cell Physiol. 176 (1998) 235–244.
[13] K. Tanaka, M. Abe, Y. Sato, Roles of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase in the signal transduction of basic fibroblast growth factor in endothelial cells during angiogenesis, Jpn. J. Cancer Res. 90 (1999) 647–654.
[14] S. Kanno, N. Oda, M. Abe, Y. Terai, M. Ito, N. Shitara, K. Tabayashi, M. Shibuya, Y. Sato, Roles of two VEGF receptors, Flt-1 and KDR, in the signal transduction of VEGF effects in human vascular endothelial cells, Oncogene 19 (2000) 2138–2146.
[15] Y. Sato, M. Abe, K. Tanaka, C. Iwasaka, N. Oda, S. Kanno, M. Oikawa, T. Nakano, T. Igarasi, Signal transduction and transcriptional regulation of angiogenesis, Adv. Exp. Med. Biol. 476 (2000) 109–115.
[16] N. Oda, M. Abe, Y. Sato, ETS-1 converts endothelial cells to the angiogenic phenotype by inducing the expression of matrix metalloproteinases and integrin β3, J. Cell Physiol. 178 (1999) 121–132.
[17] T. Nakano, M. Abe, K. Tanaka, R. Shineha, S. Satomi, Y. Sato, Angiogenesis inhibition by transdominant mutant ets-1, J. Cell. Physiol. 184 (2000) 255–262.
[18] K. Teruyama, M. Abe, C. Iwasaka-Yagi, T. Nakano, S. Takahashi, S. Yamada, Y. Sato, Role of transcription factor Ets-1 in the apoptosis of human vascular endothelial cells, J. Cell Physiol. 188 (2001) 243–252.
[19] K. Teruyama, M. Abe, T. Nakano, S. Takahashi, S. Yamada, Y. Sato, Neuropilin-1 is a downstream target of transcription factor Ets-1 in human umbilical vein endothelial cells, FEBS Lett. 504 (2001) 1–4.
[20] M. Oikawa, M. Abe, H. Kurosawa, W. Hida, K. Shirato, Y. Sato, Hypoxia induces transcription factor ETS-1 via the activity of hypoxia inducible factor-1, Biochem. Biophys. Res. Commun. 289 (2001) 39–43.
[21] S.J. Mandriota, M.S. Pepper, Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia, Circ. Res. 83 (1998) 852–859.
[22] M. Abe, Y. Sato, cDNA microarray analysis of the gene expression profile of VEGF-activated human umbilical vein endothelial cells, Angiogenesis 4 (2001) 289–298.
[23] N.W. Gale, G. Thurston, S.F. Hackett, R. Renard, Q. Wang, J. McClain, C. Martin, C. Witte, M.H. Witte, D. Jackson, C. Suri, P.A. Campochiaro, S.J. Wiegand, G.D. Yancopoulos, Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1, Dev. Cell 3 (2002) 411–423.
[24] A. Stratmann, W. Risau, K.H. Plate, Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis, Am. J. Pathol. 153 (1998) 1459–1466.
[25] R.K. Jain, Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy, Nat. Med. 7 (2001) 987–989.
[26] T. Hawighorst, M. Skobe, M. Streit, Y.K. Hong, P. Velasco, L.F. Brown, L. Riccardi, B. Lange-Asschenfeldt, M. Detmar, Activation of the tie2 receptor by angiopoietin-1 enhances tumor vessel maturation and impairs squamous cell carcinoma growth, Am. J. Pathol. 160 (2002) 1381–1392.
[27] L. Zhang, N. Yang, J.W. Park, D. Katsaros, S. Fracchioli, G. Cao, A. O'Brien-Jenkins, T.C. Randall, S.C. Rubin, G. Coukos, Tumor-derived vascular endothelial growth factor up-regulates angiopoietin-2 in host endothelium and destabilizes host vasculature, supporting angiogenesis in ovarian cancer, Cancer Res. 63 (2003) 3403–3412.
[28] Y. Sato, Role of ETS family transcription factors in vascular development and angiogenesis, Cell Struct. Funct. 26 (2001) 19–24.
[29] I.G. Maroulakou, T.S. Papas, J.E. Green, Differential expression of ets-1 and ets-2 proto-oncogenes during murine embryogenesis, Oncogene 9 (1994) 1551–1565.
[30] J. Fujimoto, I. Aoki, H. Toyoki, S. Khatun, T. Tamaya, Clinical implications of expression of ETS-1 related to angiogenesis in uterine cervical cancers, Ann. Oncol. 13 (2002) 1598–1604.
[31] J. Fujimoto, I. Aoki, H. Toyoki, S. Khatun, T. Tamaya, Clinical implications of expression of ETS-1 related to angiogenesis in uterine endometrial cancers, Ann. Oncol. 13 (2002) 1605–1611.
[32] K. Tokuhara, Y. Ogata, M. Nakagawa, K. Shirouzu, Ets-1 expression in vascular endothelial cells as an angiogenic and prognostic factor in colorectal carcinoma, Int. Surg. 88 (2003) 25–33.
[33] P.N. Span, P. Manders, J.J. Heuvel, C.M. Thomas, R.R. Bosch, L.V. Beex, C.G. Sweep, Expression of the transcription factor Ets-1 is an independent prognostic marker for relapse-free survival in breast cancer, Oncogene 21 (2002) 8506–8509.