HEX Acts as a Negative Regulator of Angiogenesis by Modulating the Expression of Angiogenesis-Related Gene in Endothelial Cells In Vitro

Tomowaki Nakagawa, Mayumi Abe, Tohru Yamazaki, Hiroki Miyashita, Hitoshi Niwa, Shoichi Kokubun, Yasufumi Sato

Objective—The hematopoietically expressed homeobox (HEX) is transiently expressed in endothelial cells (ECs) during vascular formation in embryo. Here, we investigated whether HEX played any role in angiogenesis-related properties of ECs in vitro.

Methods and Results—We transiently overexpressed HEX in human umbilical vein ECs (HUVECs). To our surprise, HEX completely abrogated the response of HUVECs to vascular endothelial growth factor (VEGF) with regard to proliferation, migration, and invasion and abolished network formation by HUVECs on Matrigel. cDNA microarray analysis and quantitative real-time reverse transcription–polymerase chain reaction combined with Western blotting revealed that HEX significantly repressed the expression of VEGF receptor-1, VEGF receptor-2, neuropilin-1, tyrosine kinase with Ig and EGF homology domains (TIE)-1, TIE-2, and the integrin αv subunit, whereas it augmented the expression of endoglin in HUVECs. We established murine embryonic stem cells that were stably transfected with HEX sense cDNA or antisense cDNA, and we examined the in vitro differentiation to ECs. Although the expression of VEGF receptor-2 was decreased in sense transfectants, the number of cells expressing VE-cadherin, a specific marker of ECs, was not altered.

Conclusions—Our present results suggest that HEX may not affect the differentiation of ECs but acts as a negative regulator of angiogenesis. (Arterioscler Thromb Vasc Biol. 2003;23:231-237.)

Key Words: angiogenesis ■ HEX ■ transcription factor ■ endothelial cell

Angiogenesis plays an essential role in a wide range of physiological as well as pathological states, including embryogenesis, wound healing, diabetic retinopathy, rheumatoid arthritis, and growth of solid tumors. Angiogenesis includes the following sequential steps: detachment of mural pericytes (vascular destabilization), degradation of the basement membrane by endothelial proteases, migration of endothelial cells (ECs), proliferation of ECs, tube formation by ECs, and reattachment of pericytes for vascular maturation.1 Numerous factors are reported to be involved in angiogenesis. Of these, EC tropic factors and their corresponding receptors have been extensively studied, including vascular endothelial growth factor (VEGF), VEGF receptor-1 (VEGFR-1/Flt-1), VEGFR-2 (KDR/Flk-1), angiopeptins, and tyrosine kinase with Ig and EGF homology domains (TIE)-2 receptor. VEGF stimulates protease synthesis in ECs and promotes EC migration and proliferation.2 Angiopoietin-1 is the agonistic ligand of the TIE-2 receptor, and it regulates the attachment of pericytes around vessels.3 Angiopoietin-2 is the antagonistic ligand of TIE-2, and it acts in opposition to angiopoietin-1.4 ECs express a variety of transcription factors during vascular formation.5 Homeobox gene products comprise a broad family of transcription factors that all contain a conserved 60–amino acid homeodomain, which binds DNA in a sequence-specific manner. Although many homeobox genes are arranged in clusters, some are located outside these regions. It has been previously demonstrated that clustered homeobox genes such as HOXB and HOXD are expressed in ECs and regulate angiogenesis in distinct manners. HOXB promotes the invasive behavior of ECs in response to angiogenic stimulation, whereas HOXD, promotes capillary morphogenesis.6,7

The hematopoietically expressed homeobox (HEX) protein, also known as the prolinc-rich homeodomain protein, is a member of the nonclustered homeodomain proteins and was initially isolated from murine hematopoietic tissue.8–10 HEX is expressed in the anterior visceral endoderm and rostral definitive endoderm in early mouse embryos and is later expressed in the liver, thyroid, and endothelial progenitors.11 Targeted disruption of the HEX gene in mouse embryos...

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revealed that HEX is essential in the endoderm for normal development of the forebrain, liver, and thyroid gland.\textsuperscript{12} In contrast, ectopic expression of Xenopus HEX gene in frog embryos\textsuperscript{13} or zebrafish HEX gene in zebrafish embryos\textsuperscript{14} caused increased numbers of ectopic ECs, suggesting that HEX played some roles in embryonic vascular development.

Here we examined whether HEX played any role in the regulation of angiogenesis. For this purpose, we modulated the expression of HEX in human umbilical vein endothelial cells (HUVECs) or murine embryonic stem (ES) cells and analyzed the angiogenic activity of ECs, the expression of angiogenesis-related genes, and the differentiation of ECs. Our results indicate that HEX does not affect the differentiation of ECs but negatively regulates angiogenesis.

**Methods**

**Cell Culture**

HUVECs were obtained from Kurabo and routinely cultured on type I collagen–coated dishes (Iwaki) in endothelial basal medium containing EC growth supplements (Clonetics Corp) and 10% fetal calf serum (FCS, JRH Biosciences). The murine ES cell line MG1.19\textsuperscript{15} was cultured in Glasgow minimum essential medium (Sigma) containing 10% FCS, 10^{-4} mol/L 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd), 1000 U/mL leukemia inhibitory factor (LIFlife Technologies, Inc), and 100 μg/mL geneticin (G418, Life Technologies, Inc). OP9 cells\textsuperscript{16} were cultured in minimum essential alpha medium (αMEM) containing 20% FCS.

**Transfection of HEX cDNA in ECs**

Murine HEX cDNA was kindly provided by Dr Wiedemann (Chester Beatty Laboratories, Institute of Cancer Research, London, UK). The adenovirus vector expression kit (Takara) was used, and replication-deficient recombinant adenoviruses were constructed according to the COS/TPC method.\textsuperscript{17} AdHEX was obtained by in vivo homologous recombination between the transfer cassette bearing the HEX expression unit and almost the entire adenovirus genome, and the gous recombination between the transfer cassette bearing the HEX expression unit and almost the entire adenovirus genome, and the restriction enzyme–digested adenovirus genome was tagged with terminal protein in 293 cells.\textsuperscript{18} ECs were infected with adenovirus vectors at indicated multiplicities of infection (MOIs). Adenoviral infection was carried out in serum-free medium 199 (Nissui) for 1 hour at 37°C.

**Cell Migration**

Migration of cells was examined by using the wound assay as previously described.\textsuperscript{19} HUVECs were transfected with AdHEX or Adnull. After 48 hours, confluent cell monolayers were wounded with a razor blade, and the medium was changed to medium 199 containing 5% FCS with or without 1 nmol/L VEGF (R&D Systems, Inc). After 24 hours of incubation, cells that had migrated across the edge of the wound and into the gap were counted as migrating cells.

**Cell Invasion**

Cell invasion assays were performed by using a Matrigel-coated invasion chamber equipped with an 8-μm-pore-size micropore filter (BioCoat Matrigel invasion chamber; 24 wells; 8.0-μm pore size; Becton Dickinson). The lower compartment of the invasion chamber was filled with medium 199 containing 5% FCS with or without 1 nmol/L VEGF. After the adenovirus transfection, HUVECs were harvested, resuspended in medium 199 containing 5% FCS with or without 1 nmol/L VEGF, and inoculated into the upper chamber (2.5×10^5 cells per chamber). After incubation at 37°C for 24 hours, the Matrigel on the filter was scraped off, and the filter was fixed with methanol and stained with hematoxylin. Cells that had penetrated the filter were then counted.

**DNA Synthesis**

DNA synthesis was examined by 5′-bromo-2′-deoxyuridine (Brdu) incorporation and chemiluminescence detection (Roche Diagnostics). After the adenovirus transfection, 2000 HUVECs were inoculated into each well of a 96-well multiliter black plate. Cells were allowed to adhere for 24 hours in endothelial basal medium with 10% FCS and then starved in medium 199 with 5% FCS for 6 hours. After starvation, cells were incubated with or without 1 nmol/L VEGF, and 10 μL of 10× BrdU was added to each well. After 24 hours of incubation, BrdU chemiluminescence was analyzed by ELISA.

**Network Formation by HUVECs on Matrigel**

After the adenovirus transfection, 3×10^5 HUVECs were inoculated on a Matrigel-coated dish (Becton Dickinson) and incubated in medium 199 with 5% FCS for 8 hours. Network formation was observed by phase-contrast microscopy.

**cDNA Microarray Analysis**

Total RNA was prepared from HUVECs infected with AdHEX or Adnull with the use of Isogen (Nippongene), and poly(A)+ RNA was purified from the total RNA by using an Oligotex-dt30 mRNA purification kit (Takara). cDNA probe synthesis, hybridization with a human UniGEM V cDNA microarray, and signal analysis were conducted by Genome Systems (IncyteGenomics, St. Louis, Mo).

**Quantitative RT-PCR Analysis**

Total RNA samples were prepared from HUVECs transfected with AdHEX or Adnull by using Isogen. First-strand cDNA was generated by using the first-strand cDNA synthesis kit for reverse transcription–polymerase chain reaction (RT-PCR, Roche Diagnostics). Quantitative RT-PCR was performed with a LightCycler system (Roche Diagnostics) according to the manufacturer’s instructions. The amount of a cDNA product was measured as a fluorescence signal proportional to the amount of the specific target sequence present. The sense and antisense primer pairs used were as follows: VEGFR-1, GAGCATCAGCAGCATGCGCAG, GATCTTG-TATTAACGTTCGCAG; VEGFR-2, GTTTTGACTGAAGACGAGCTTGG, ACTATAGATGTTGACCCCGAG; TIE-1, AGGTC-ACGCTTGGCGGCTT, CCAAAACGGCCCTCTCTGCTT; TIE-2, TAGAGCGTGGAACACAGCTACAG, CTATTGGCAATTGCAAAATGCGG; ephrinB2, GCCGACACAGGCACCAGGACTA, GCCGACACAGGCACCAGGACTA; GATC, CACCACCTGACCAAGAGACAGG, ATGTCTGTGCTCCCTGATCTT; matrix metalloproteinase (MMP)-1, GACAGATCTCATGCGG-CAC, GTGCGCAATTACGAGAGAAAGT; tissue inhibitor of metalloproteinase (TIMP)-1, CACCACAGACCCAGGATGGGATTGG; ELISA.

**Western Blot Analysis**

HUVECs were transfected with AdHEX or Adnull. After 48 hours, total protein was extracted with modified radioimmunoprecipitation assay (RIPA) buffer as previously described.\textsuperscript{20} Equal amounts of samples were applied to SDS–polyacrylamide gel electrophoresis gels under reducing conditions and then transferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech). Membranes were blocked and probed with primary antibodies. Signals were visualized by using horseradish peroxidase–conjugated sec-
secondary antibodies and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) with an LAS-1000 image analyzer (Fuji).

Antibodies against VEGFR-1, VEGFR-2, TIE-1, TIE-2, and neuropilin-1 were obtained from Santa Cruz Biotechnology, Inc, and anti-integrin αv antibody was from Chemicon International Inc. Rabbit polyclonal anti-endoglin antibody was a gift from Dr K. Miyazono (Graduate School of Medicine, University of Tokyo, Tokyo, Japan).

Differentiation of ES Cells to ECs In Vitro

To constantly maintain expression of introduced genes in ES cells, we used the supertransfection system. In brief, pHPCAG containing the gene to be expressed in ES cells was transfected in MG1.19, which was the CCE ES cell line carrying the primary episomal pMGD20neo for supertransfection. pHPCAG contains a CAG promoter, which is highly active not only in ECs but also in a wide range of cell types. The gene introduced into pHPCAG is expressed by large T from pMGD20neo in MG1.19 cells cultured with hygromycin in addition to geneticin. Murine HEX cDNA was introduced into pHPCAG in a sense (pHPCAG-m-HEX-S) or an antisense (pHPCAG-m-HEX-AS) orientation. MG1.19 cells were transfected with pHPCAG (mock), pHPCAG-m-HEX-S, or pHPCAG-m-HEX-AS by using Lipofectamine Plus (Life Technologies, Inc). Transfectants were selected by addition of 100 μg/mL hygromycin B (Life Technologies, Inc).

To induce differentiation, 1.4 × 10^4 cells/10-cm dish of MG1.19 cells transfected with mock, pHPHPCAG-m-HEX-S, or pHPHPCAG-m-HEX-AS were cultured on confluent OP9 cells (a gift from Dr H. Kodama, RIKEN, Wako, Japan) in MEM supplemented with 10% FCS and 5 × 10^-5 mol/L 2-mercaptoethanol in the absence of LIF. On days 3, 4, and 5, the effect of HEX on the differentiation of ES cells toward EC lineage was examined by flow cytometry as described previously. The cultured cells were harvested by incubation in cell-dissociation buffer (Life Technologies, Inc). The harvested cells were incubated in mouse serum for 30 minutes on ice to block nonspecific antibody binding and then incubated with allophycocyanin-conjugated anti-mouse CD144/VE-cadherin antibody and R-phycoerythrin–conjugated anti-mouse Flk-1/VEGFR-2 antibody (Avast2×1) for 15 minutes on ice. Living cells excluding propidium iodide (Sigma) were analyzed by FACS vantage (Becton Dickinson).

Immunohistochemical Analysis of HEX In Vivo

Male C57BL/6 mice were used at 4 weeks of age. Growth factor–reduced Matrigel (500 μL, Collaborative Biomedical Products) containing 8.3 nmol/L basic fibroblast growth factor (Collaborative Biomedical Products) in liquid form at 4°C was injected into...
abdominal subcutaneous tissues at the midperitoneal area of the mice. On day 5 after injection, the mice were humanely killed after they were anesthetized with ether, and the gel was recovered. Matrigel gels were then fixed in 4% paraformaldehyde containing phosphate-buffered saline and embedded in paraffin. Gel sections (3 μm thick) were processed for immunohistochemistry by using the ABC method. Rabbit polyclonal anti-human HEX antibody (a gift from Dr Nagai, Graduate School of Medicine, University of Tokyo, and Dr Kurabayashi, Gunma University School of Medicine), anti-mouse platelet endothelial cell adhesion molecule (PECAM)-1/CD31 antibody (Santa Cruz Biotechnology, Inc), and normal rabbit IgG fraction (Vector Laboratories, Inc) were used as primary antibodies.

Calculations and Statistical Analysis
The statistical significance of differences in the data were evaluated by an unpaired ANOVA, and probability values were calculated by Student’s t test. A probability value <0.05 was considered statistically significant.

Results

Effect of HEX on Angiogenesis-Related Properties of HUVECs
We observed HEX protein expression in ECs at the site of postnatal angiogenesis in vivo. Nuclear HEX protein staining was demonstrated in the ECs of neovessels (online supplementary material 1; see http://www.atvb.ahajournals.org). Therefore, we examined the function of HEX in vitro in the present study. To do so, we constructed an adenoviral vector encoding HEX cDNA (AdHEX) fused to a polyhistidine tag. The efficacy of the adenovirus-mediated gene transfer was confirmed by infecting HUVECs with different titers of AdHEX. Quantitative RT-PCR revealed that HEX mRNA expression was MOI dependent, and all of the cell nuclei were positive for anti-His6 antibody immunostaining at an MOI of 200 (online supplementary material 2; see http://www.atvb.ahajournals.org). The expression levels at an MOI of 50 were almost equal to that of WEHI, a myelomonocytic leukemia cell line that endogenously expresses HEX.8 We infected HUVECs with AdHEX or the control Adnull and examined their effects on angiogenesis-related properties of HUVECs. Angiogenesis requires the migration, invasion, and proliferation of ECs, and these processes are stimulated by VEGF. Whereas basal migration levels were almost equal between Adnull- and AdHEX-infected HUVECs, the response to VEGF was lost in HUVECs infected with AdHEX (Figure 1A). AdHEX inhibited the VEGF-stimulated migration of HUVECs in an MOI-dependent manner, and significant inhibition was detected at an MOI as low as 10 (Figure 1B). Cell invasion is a consequence of cell adhesion, cell migration, and matrix degradation and is essential for the initiation of sprouting angiogenesis. Invasion chamber experiments showed that HEX inhibited VEGF-induced cell invasion of HUVECs (Figure 1C). Similar to the results of migration and invasion, whereas basal levels of DNA synthesis were almost equal between Adnull- and

<table>
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<tr>
<th>Gene Name</th>
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<tr>
<td>VEGFR-1</td>
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*Real-time RT-PCR.
AdHEX-infected HUVECs, VEGF responsiveness was lost in AdHEX-infected HUVECs (Figure 1D). ECs spontaneously form networks when plated on Matrigel. Whereas Adnull-infected HUVECs formed network structures on Matrigel surfaces, this network-forming ability was lost in HUVECs infected with AdHEX, and they formed cell aggregates (Figure 2). These results indicate that HEX decreases the angiogenic activities of HUVECs.

Role of HEX in the Expression of Angiogenesis-Related Genes in HUVECs

Because HEX has transcription factor characteristics, its expression might alter the angiogenic properties of HUVECs by modulating the expression of angiogenesis-related genes. To determine the role of HEX in HUVEC gene transcription, we compared the mRNA expression profile of Adnull- and AdHEX-infected HUVECs by using a combination of cDNA microarray analysis and quantitative real-time RT-PCR. The Table summarizes the relative expression of individual genes. We observed no contradictions in terms of gene expression of VEGFR-1 mRNA between cDNA microarray and quantitative RT-PCR analyses. Expression of receptor and membrane protein genes, such as VEGFR-1, VEGFR-2, TIE-1, TIE-2, and neuropilin-1, were repressed <0.5-fold in AdHEX-infected HUVECs (Table 1). A time-course experiment revealed that decreased levels of VEGFR-2 were evident as early as 12 hours after infection (Figure 3A). Decreased VEGFR-2 mRNA levels were detected at an MOI as low as 10 and were maximal at an MOI of 50 (Figure 3B). In contrast, mRNA levels of endoglin, ephrin B2, urokinase-type plasminogen activator, tissue-type plasminogen activator, plasminogen activator inhibitor-1, TIMP-1, TIMP-2, and TIMP-3 were upregulated 2-fold (Table 1).

Protein levels of VEGFR-1, VEGFR-2, neuropilin-1, integrin αv, and endoglin were further analyzed by Western blotting. As shown in Figure 4, protein levels of VEGFR-1, VEGFR-2, neuropilin-1, TIE-1, TIE-2, and integrin αv were decreased, whereas that of endoglin was increased. These changes correlated very well to those of mRNA levels.

Effects of HEX on the Differentiation of ES Cells to ECs

The ectopic expression of Xenopus HEX gene in frog embryos or zebrafish HEX gene in zebrafish embryos caused increased numbers of ectopic endothelial progenitors or ECs. Here we examined whether HEX affected the differentiation of ECs. For this purpose, we transfected HEX sense cDNA or antisense cDNA to the murine ES cell line MG1.19 as described in Methods. Quantitative RT-PCR analysis revealed that HEX mRNA was expressed in MG1.19 cells, and its level was increased by 1.4-fold after 5 days’ cultivation on OP9. Northern blot analysis further revealed that the expression of HEX mRNA could be detected in sense transfecant but not in mock or antisense transfecants (data not shown). The expression of VE-cadherin, a specific marker of ECs, became apparent on day 5 in each transfecant, but HEX did not lead to any alteration in the number of VE-cadherin–expressing cells (Figure 5A). The expression of HEX in ES cells resulted in reduction of VEGFR-2 expression on days 4 and 5 (Figure 5B). Among VE-cadherin–positive cell populations at day 5, 14% of sense transfecants were VEGFR-2–negative, whereas 7% of antisense transfecants were VEGFR-2–negative.

Discussion

In the current report, we have demonstrated for the first time that the HEX homeobox protein significantly reduces the expression of genes, including VEGFR-1, VEGFR-1, TIE-1, TIE-2, and neuropilin-1, and abrogates the angiogenic properties of ECs. Our in vitro experiments suggest that HEX acts as a negative regulator of angiogenesis. However, it remains to be clarified whether HEX has the same effects in vivo.

The precise mechanism of how HEX represses the expression of those genes remains to be elucidated. However, HEX...
is known to act as a transcriptional repressor in liver cells, ES cells, and hematopoietic cells.\textsuperscript{24–26} Generally, mechanisms of transcriptional repression can be classified into 4 main categories: steric hindrance, quenching, direct repression, and modulation of chromatin structure. Guiral et al\textsuperscript{26} have recently shown that HEX repressed transcription from TATA box-containing promoters by binding to the TATA box and sterically hindering the binding of TATA box–binding proteins. Because the N-terminal proline-rich domain of HEX appears to be responsible for this repressor activity, it is therefore possible that HEX represses the expression of angiogenesis-related receptors and membrane proteins in HUVECs by steric hindrance.

We further examined the effect of HEX on the differentiation of ECs. For this purpose, we applied a murine ES cell in vitro differentiation system, and we evaluated the differentiation of ECs by analyzing the appearance of VE-cadherin–positive cells. In this connection, HEX did not affect the expression of VE-cadherin mRNA in HUVECs (Table). It was previously described that ectopic expression of \textit{Xenopus HEX} gene in frog embryos\textsuperscript{13} or zebrafish \textit{HEX} gene in zebrafish embryos\textsuperscript{14} caused increased numbers of ECs. However, our results indicate that HEX does not significantly alter the number of differentiated ECs (Figure 5). We assume that ectopic \textit{HEX} gene transfection experiments might have modulated expression in cells other than ECs or its progenitors, which then indirectly affected the differentiation or proliferation of ECs.

Because a variety of transcription factors are expressed in ECs during vascular development or angiogenesis, the cross-regulation of transcription factors is important. Although HEX has been reported to induce SCL/tal-1 expression in zebrafish embryos,\textsuperscript{14} we observed no increases in SCL/tal-1 mRNA levels in HUVECs after infection with Ad\textit{HEX} (Table). However, we did observe HEX-mediated repression of Runx-1/PEBP2αA gene in HUVECs. We have previously reported that Runx-1/PEBP2αA is induced in ECs in response to VEGF or basic fibroblast growth factor and is required for angiogenesis.\textsuperscript{27} Therefore, the decreased expression of Runx-1/PEBP2αA may, in part, explain the negative role of HEX in angiogenesis.

The function of HEX may not be simple, because our cDNA microarray analysis revealed that HEX simultaneously increased the expression of various genes. Indeed, HEX can act as a transcription activator as well.\textsuperscript{28} Among various genes of increased expression, endoglin is a binding site of transforming growth factor β\textsuperscript{29} and is highly expressed in ECs during angiogenesis.\textsuperscript{30} Endoglin is involved in vascular maturation, because targeted disruption of the endoglin gene in mice exhibited defective vascular maturation, poor smooth muscle cell accumulation, and arrested endothelial remodeling.\textsuperscript{31} Consequently, our results may suggest that HEX not only acts as a negative regulator of angiogenesis but also promotes vascular maturation by inducing endoglin in ECs.

\textbf{Acknowledgments}

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\textbf{References}


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