Hypoxia-Inducible Factor-1 Mediates Activation of Cultured Vascular Endothelial Cells by Inducing Multiple Angiogenic Factors


Abstract—Hypoxia-inducible factor-1 (HIF-1) mediates transcriptional activation of vascular endothelial growth factor (VEGF) and other hypoxia-responsive genes. Transgenic expression of a constitutively stable HIF-1α mutant increases the number of vascular vessels without vascular leakage, tissue edema, or inflammation. This study aimed to investigate the molecular basis by which HIF-1 mediates the angiogenic response to hypoxia. In primary human endothelial cells, hypoxia, desferrioxamine, or infection with Ad2/HIF-1α/VP16, an adenoviral vector encoding a constitutively stable hybrid form of HIF-1α, increased the mRNA and protein levels of VEGF, angiopoietin-2 (Ang-2), and angiopoietin-4 (Ang-4). Infection with Ad2/CMVEV (a control vector expressing no transgene) had no effect. Angiopoietin-1 (Ang-1) expression was not detected in human endothelial cells. Ang-4 was also induced by hypoxia or Ad2/HIF-1α/VP16 in human cardiac cells, whereas Ang-1 expression remained unchanged. Recombinant Ang-4 protein protected endothelial cells against serum starvation-induced apoptosis and increased cultured endothelial cell migration and tube formation. Ad2/HIF-1α/VP16 stimulated endothelial cell proliferation and tube formation. Hypoxia- or Ad2/HIF-1α/VP16-induced tube formation was significantly reduced by a Tie-2 inhibitor. These results suggest that HIF-1 mediates the angiogenic response to hypoxia by upregulating the expression of multiple angiogenic factors. Ang-4 can function similarly as Ang-1 and substitute for Ang-1 to participate in hypoxia-induced angiogenesis. Activation of the angiopoietin/Tie-2 system may play a role in the ability of HIF-1 to induce hypervascularity without excessive permeability. (Circ Res. 2003;93:664-673.)

Key Words: hypoxia-inducible factor-1 ■ hypoxia ■ angiogenesis ■ angiopoietins ■ vascular endothelial growth factor

Administration of a single growth factor in the form of protein or gene has been shown to promote tissue neovascularization in animal models and patients. However, transgenic overexpression of vascular endothelial growth factor (VEGF) alone in mice results in increased numbers of primarily leaky vascular vessels with tissue edema and inflammation, suggesting that VEGF needs to work in conjunction with other angiogenic factors to produce a healthy vasculature. In a variety of conditions, such as malignant tumors, wound healing, and myocardial ischemia, hypoxia is a fundamental stimulus for angiogenesis. Activation of hypoxia-responsive genes including VEGF is mediated by hypoxia-inducible factor-1 (HIF-1), a heterodimeric basic helix-loop-helix-PAS domain transcription factor. HIF-1 is composed of two subunits, HIF-1α and HIF-1β (aryl hydrocarbon nuclear translocator). Whereas the β-subunit protein is constitutively present, the stability of the α-subunit and its transcriptional activity are precisely controlled by the intracellular oxygen concentration. Knocking-out the HIF-1α alleles in mice results in embryonic lethality with vascular regression. Overexpression of a DNA plasmid encoding HIF-1α/VP16, a constitutively stable hybrid of HIF-1α, increases angiogenesis and blood supply in animal models of ischemic hindlimb and myocardium. Recent studies suggest that transgenic expression of a constitutively stable HIF-1α mutant in mice results in increased numbers of vascular vessels without the excessive permeability observed in transgenic mice overexpressing VEGF. The underlying molecular mechanism for the differences in the quality of the vasculature resulting from overexpression of VEGF or HIF-1α is not understood.

Angiopoietins are ligands for the endothelium-specific receptor tyrosine kinase Tie-2. Angiopoietin-1 (Ang-1) plays an important role in the assembly of newly formed...
vasculature and in the maintenance of vascular integrity. Transgenic mice overexpressing a combination of VEGF and Ang-1 exhibit marked induction of hypervascularity without excessive permeability, highlighting the importance of Ang-1 in the formation of normal vessels. Angiopoietin-2 (Ang-2) serves as a natural antagonist of Ang-1. The role of Ang-2 in angiogenesis is highly dependent on the presence of other angiogenic factors, particularly VEGF. Ang-2 antagonizes the activation of Tie-2 by Ang-1 and causes endothelial cell apoptosis and vascular regression. In the presence of VEGF, however, Ang-2 destabilizes the preexisting vasculature and consequently makes it more responsive to angiogenic stimuli. When coadministered with VEGF, both Ang-1 and Ang-2 are capable of augmenting angiogenesis. Recently, human angiopoietin-4 (Ang-4) was cloned. Ang-4 binds to Tie-2 as an agonist, although its precise function has not been documented. Hypoxia has been shown to regulate the expression of angiopoietins and Tie-2, suggesting their participation in the angiogenic response to hypoxia. However, it is unknown whether HIF-1 mediates hypoxic induction of angiopoietins. It is also not clear if Ang-4 participates in the angiogenic response to hypoxia.

This study aimed to elucidate the role of HIF-1 in the angiogenic response to hypoxia. We demonstrated that in primary human artery endothelial cells, desferrioxamine and adenovirus-mediated expression of HIF-1α/VP16 mimicked the hypoxic induction of multiple angiogenic factors including VEGF, Ang-2, and Ang-4. HIF-1α/VP16 also stimulated tube formation of cultured endothelial cells, which was partially inhibited by a VEGF receptor 2 or Tie-2 inhibitor. Our results suggest that HIF-1 mediates the angiogenic response to hypoxia by activating both the VEGF and angiopoietin/Tie-2 system. Activation of the angiopoietin/Tie-2 system by HIF-1, in particular the upregulation of Ang-4 that was shown to function similarly as Ang-1, may explain the difference in the quality of the vasculature resulting from overexpression of VEGF or HIF-1α.

Materials and Methods

Construction of Adenoviral Vectors
Ad2/HIF-1α/VP16 encoding HIF-1α/VP16, Ad2/CMVβgal encoding β-galactosidase, and Ad2/CMVEV expressing no transgene were generated as described previously. The HIF-1α/VP16 hybrid, constructed by truncating the transactivation and oxygen-dependent degradation domains of HIF-1α and then joining the HSV VP16
transactivation domain fragment downstream, was stable under normoxic conditions. Ad2/HIF-1α/DN was constructed as Ad2/HIF-1α/VP16, except it encodes a dominant-negative form of HIF-1α with the deletions of the DNA binding (aa 4 to 27) and transactivation domains (aa 390 to 820). 38

Cell Culture
Human fetal cardiac cells (passaged twice) obtained from Clonetics (San Diego, Calif) were cultured as described previously. 37 Human coronary (HCAECs) or pulmonary artery endothelial cells (HPAECs) were also purchased from Clonetics and cultured using the optimized growth medium (basal medium [EBM] supplemented with 2% or 4% [vol/vol] fetal bovine serum [FBS] and SingleQuots [epidermal growth factor, hydrocortisone, VEGF, basic fibroblast growth factor, insulin growth factor-1, ascorbic acid, heparin, gentamycin, and amphoterin B]) developed by Clonetics. Hypoxia was created by placing the cells in a hypoxia chamber (1% O2, 5% CO2, 37°C) for 18 or 24 hours.

Generation of Conditioned Medium
HPAECs were infected with adenoviral vectors for 4 hours, washed three times with PBS, and finally maintained in fresh EBM containing 2% FBS for an additional 20 hours. The conditioned medium was then collected and filtered using a 0.2-μm filter.

Measurement of Endothelial Cell Caspase-3 Activity, DNA Fragmentation, Apoptosis, Proliferation, Wound Healing, Permeability, and Tube Formation
HPAECs maintained in EBM supplemented with or without 2% FBS were treated with recombinant human proteins (R&D systems) or infected with adenoviral vectors. Caspase-3 activity and DNA fragmentation were measured using the Apo-ONE Homogeneous Caspase-3/−7 Assays kit (Promega) and Cell Death ELISA kit (Roche), respectively, according to manufacturer’s manual. Apoptosis was detected using Hoechst 33258 staining (see expanded Materials and Methods section in the online data supplement available at http://www.circresaha.org). The number of cells was counted using a hemocytometer. Endothelial cell migration was estimated using a monolayer denudation assay, as described previously with minor modifications. 39 Wounded HPAEC cultures were maintained in EBM supplemented with 4% FBS. The cell-free area was photographed 12 hours later and analyzed using ImageJ, an image analysis software developed by the NIH.

Fully confluent HPAEC monolayers cultured on fibronectin-coated transwell (0.4-μm pore size, BD Bioscience) with EBM containing 2% FBS were treated with Ang-1, Ang-4, or VEGF for 2 hours. FITC-dextran (1 mg/mL, average molecular weight 40 000; Sigma) was added into the upper compartment of the transwell cultures, followed by stimulation with thrombin (1 U/mL, Sigma). The amount of FITC-dextran in the culture medium (50 μL) taken from the lower compartment, indicative of the permeability of the HPAEC monolayer, was then determined using a fluorometer.

The angiogenic response to various treatments was assessed using an in vitro capillary/tube-like structure formation assay. Primary human endothelial cells were seeded on 24-well cell culture plates precoated with Matrigel (BD Bioscience) at 104 cells/well and maintained in EBM under various conditions. The capillary/tube-like structures were visualized by light microscopy 8 hours later and analyzed using ImageJ.

Measurement of Gene Expression
The mRNA levels of specific genes were measured by TaqMan 5′ nuclease fluorogenic quantitative PCR analysis and normalized to 18S rRNA. 37 Primers and probes (online Table 1, available in the online data supplement at http://www.circresaha.org) were designed according to ABI-Perkin Elmer guidelines. The protein levels of VEGF and Ang-2 in the supernatant were measured using ELISA kits from R&D Systems, whereas an Ang-4 ELISA was developed using monoclonal and polyclonal antibodies against human Ang-4 from R&D Systems.

Statistical Analysis
Data (mean±SEM) were analyzed by one-way ANOVA, followed by a modified Student’s t test. The number of samples examined is indicated by n. A value of P < 0.05 was considered statistically significant.

Results
Effects of Hypoxia, Desferrioxamine, and Ad2/HIF-1α/VP16 on Expression of Angiogenic Factors and Their Receptors in Cultured Human Endothelial Cells
In HCAECs maintained under normoxic conditions, low levels of VEGF, VEGFR1, VEGFR2, Ang-2, Ang-4, and Tie-2 mRNA were detected by TaqMan analysis. As previously described, 29–35 hypoxia significantly increased VEGF and Ang-2 mRNA levels. The mRNA levels of VEGFR1, Ang-4, placenta growth factor (PIGF), and platelet-derived growth factor (PDGF) were also upregulated (Figure 1), whereas VEGFR2 and Tie-2 expression remained unchanged.

Figure 2. Effects of hypoxia, desferrioxamine, or Ad2/HIF-1α/VP16 on the protein levels of VEGF (A), Ang-2 (B), and Ang-4 (C). HCAECs were maintained under normoxic conditions (N), subjected to hypoxia (H), treated with desferrioxamine (100 μmol/L, D), Ad2/HIF-1α/VP16 (3, 10, and 100 MOI). Separate cells were infected with Ad2/CMV-βgal (100 MOI, gal) or Ad2/HIF-1α/DN (100 MOI, DN) 24 hours before hypoxia (gal+H or DN+H). The supernatant over 24 hours...
Ang-1 was not expressed in HCAECs maintained under normoxic or hypoxic conditions (data not shown). Desferrioxamine, an iron antagonist that inhibits the prolyl hydroxylation of HIF-1α and consequently causes a well characterized "hypoxia mimetic" effect, also significantly increased the mRNA levels of VEGF, VEGFR1, Ang-2, Ang-4, PDGF, and PlGF. Furthermore, infection with Ad2/HIF-1α/VP16 increased the mRNA levels of VEGF, VEGFR1, PI GF, and Ang-4 in a dose-dependent manner (Figure 1). Interestingly, a bell-shaped dose response was observed with Ang-2 and PDGF. The increase in Ang-2 and PDGF mRNA levels by Ad2/HIF-1α/VP16 peaked at 3 MOI and then declined to basal levels at higher MOIs. Infection with Ad2/CMV/βgal had no significant effect on the mRNA levels of these genes. In another experiment, protein levels of VEGF, Ang-2, and Ang-4 in the supernatant, as measured by ELISA, were increased by hypoxia, desferrioxamine, or infection with Ad2/HIF-1α/VP16 (Figure 2). The hypoxic induction was inhibited by preinfection with Ad2/HIF-1α/DN encoding a dominant-negative form of HIF-1α, whereas Ad2/CMV/βgal had no effect. Our studies suggest that HIF-1 mediates hypoxic activation of both the VEGF and angiopoietin system. The mRNA levels of VEGF, VEGFR1, Ang-2, and Ang-4 in HPAECs were also increased by either hypoxia or infection with Ad2/HIF-1α/VP16 (Figure 3). However, hypoxic induction of PDGF and PIGF was not mimicked by Ad2/HIF-1α/VP16 in HPAECs, suggesting that in HPAECs, hypoxic induction of PDGF and PIGF may not be mediated by HIF-1.

In myocardial ischemia, hypoxic cardiomyocytes may stimulate angiogenesis by paracrine signaling. In this study, Ang-4 mRNA was detected in human fetal cardiac cells maintained under normal culture conditions (see online Figure 1, available in the online data supplement at http://www.circresaha.org). Hypoxia and infection with Ad2/HIF-1α/VP16 significantly increased Ang-4 mRNA levels by 4.5±0.3-fold (n=3; P<0.05) and 50.0±2.9-fold (P<0.01), respectively. As we previously reported, hypoxia or infection with Ad2/HIF-1α/VP16 also significantly upregulated the mRNA levels of VEGF but not Ang-1 or Ang-2 (data not shown). Taken together, these results suggest that hypoxia and expression of HIF-1α/VP16 induce multiple angiogenic factors in a tissue-specific manner. Ang-4 rather than Ang-1 may participate in the angiogenic response to hypoxia in HCAEC, HPAEC, and cardiac cells.
Effects of Ang-4 on Endothelial Cell Apoptosis, Migration, Permeability, and Tube Formation

The finding that Ang-4 but not Ang-1 was significantly induced by hypoxia or HIF-1α/VP16 in endothelial and cardiac cells prompted us to examine the function of Ang-4. Withdrawing serum and growth factors from the optimized growth medium increased the activity of the executioner caspase-3 and endothelial cell apoptosis, which were significantly inhibited by Ang-1 and Ang-4 (Figure 4). Tie-2Fc (Sigma), a Tie-2 inhibitor, reversed the inhibitory effect of Ang-4 on serum starvation-induced caspase-3 activation. These results indicate that Ang-4 protects cultured endothelial cells against serum starvation–induced apoptosis through Tie-2 activation.

To examine the effect of Ang-4 on endothelial cell migration, 90% confluent HPAEC monolayers were scraped. Wounded cultures incubated in EBM supplemented with 4% FBS in the absence of growth factors for 12 hours showed migration of cells into the denuded area, recovering part of the exposed surface. Ang-1 and Ang-4 significantly accelerated the recovery of the exposed surface area by Ang-4 abolishment by Tie-2Fc. However, Ang-1 and Ang-4 failed to stimulate endothelial cell proliferation (data not shown). These results suggest that Ang-4 is not an endothelial cell mitogen but acts to enhance endothelial cell migration.

HPAECs or HCAECs seeded on Matrigel and maintained with EBM supplemented with 2% FBS alone for 8 hours developed tube-like structures. Ang-1 and Ang-4 significantly enhanced tube formation of HPAECs, whereas Ang-2 had no effect (Figure 5B). VEGF also increased the length of the tube-like structures of HCAECs (Figure 5C). A combination of VEGF and Ang-4 showed an additive effect. In addition, Ang-1 and Ang-4 but not VEGF significantly reduced thrombin-induced increase in the permeability of endothelial cell monolayers (Figure 5D). Taken together, these results suggest that Ang-4 enhances endothelial cell tube formation and inhibits the increase in the permeability of endothelial monolayers, demonstrated functions of Ang-1.20,21,26

Effects of Ad2/HIF-1α/VP16 on Endothelial Cell Proliferation and Tube Formation

Direct Infection of HPAECs with Ad2/HIF-1α/VP16 significantly increased the number of cells in a dose-dependent manner, whereas Ad2/CMVEV had no effect (Figure 6A). The conditioned medium harvested from the donor HPAECs infected with Ad2/HIF-1α/VP16 but not Ad2/CMVEV also significantly increased the number of cells (Figure 6B). Furthermore, noninfected HPAECs seeded on Matrigel and cultured in the conditioned medium harvested from control donor cells for 6 hours developed tube-like structures (Figure 7). The tube-like structures were more extensive in cells cultured with conditioned medium harvested from donor cells infected with Ad2/HIF-1α/VP16, whereas Ad2/CMVEV had no effect. These results suggest that HIF-1α/VP16 stimulates endothelial cell proliferation and tube formation, mainly through autocrine signaling by secreted growth factors.

Effect of Soluble Tie-2 Receptors on Hypoxia- or HIF-1–Induced Tube Formation

We next assessed the involvement of the angiopoietin/Tie-2 system in hypoxia-induced endothelial cell tube formation.
HPAEC cultures on Matrigel were maintained in EBM supplemented with 2% FBS under normoxic conditions or exposed to hypoxia for 8 hours in the presence or absence of Tie-2Fc. The tube-like structures were significantly more extensive in the cultures exposed to hypoxia, compared with time-matched normoxic controls (Figure 8). This hypoxia-induced enhancement of tube formation was partially inhibited by Tie-2Fc, suggesting the contribution of the angiopoietin/Tie-2 system in hypoxia-induced tube formation. Either Tie-2Fc or a VEGFR2 kinase inhibitor I ((Z)-3-[(2,4-Dimethyl-3-(ethoxycarbonyl)-pyrrol-5-yl)methylidenyl] indolin-2-one; Calbiochem) also significantly reduced the increase in tube-like structures induced by conditioned medium from donor endothelial cells infected with Ad2/HIF-1α/VP16 (Figure 7), providing evidence that the activation of the angiopoietin/Tie-2 system contributes to HIF-1α-mediated angiogenic response to hypoxia.

**Discussion**

**Ang-4 May Function as Ang-1 and Participate in the Angiogenic Response to Hypoxia**

Human Ang-4 has been shown to bind to Tie-2, resulting in its phosphorylation. In this study, in cultured endothelial cells Ang-4 inhibited serum starvation–induced caspase-3 activation, apoptosis, and thrombin-induced increase in permeability, stimulated migration, and enhanced tube formation, indicating that Ang-4 is capable of functioning as Ang-1. Ang-1 was not expressed in human coronary and pulmonary endothelial cells maintained under control (normoxic) conditions or exposed to hypoxia. In contrast, Ang-4 mRNA was detected under control conditions and further increased by hypoxia or desferrioxamine. In fetal human cardiac cells, hypoxia also upregulated the mRNA levels of Ang-4, whereas Ang-1 expression was not increased. Furthermore, tube formation in endothelial cells exposed to hypoxia was significantly reduced by a soluble Tie-2 receptor, indicating the contribution of the angiopoietin/Tie-2 system to hypoxia-induced angiogenesis. Our results suggest that Ang-4 can substitute for Ang-1 to participate in the angiogenic response to hypoxia.

It is known that Ang-2 is highly expressed at the site of vascular remodeling and in tumor vessels. In the corneal micropocket assay of neovascularization, addition of Ang-2 to VEGF increases neovascularization, compared with VEGF alone. Hypoxia upregulates Ang-2 expression.
destabilizing the existing vascular vessels. Ang-2 as a permissive factor to facilitate angiogenesis by the hypothesis that hypoxia induces the expression of Ang-4 and desferrioxamine also coincidentally upregulated Ang-2 and Ang-4 expression in cultured human endothelial cells. At high concentrations, Ang-2 may act as an Tie-2 agonist. These observations suggest that the interaction between angiopoietins is complex. We demonstrated that enhanced tube formation in endothelial cells exposed to hypoxia was significantly reduced by a soluble Tie-2 receptor, indicating an overall activation of the Tie-2 receptor. Furthermore, Ang-4 but not Ang-2 enhanced endothelial cell tube formation under our experimental conditions, suggesting that Ang-4 rather than Ang-2 contributes to hypoxia-induced endothelial cell tube formation observed in our studies. It is possible that in vivo in the early stage of angiogenesis, downregulation of the Tie-2 system by increasing Ang-2 expression may prepare the vascular bed, whereas activation of Tie-2 by Ang-4 facilitates angiogenesis at later stages. It is also possible that Ang-2 may act as a Tie-2 antagonist and permissive factor to enhance VEGF-induced activation of endothelial cells initially and then serve as a partial Tie-2 agonist to aid Ang-4 in vascular vessel assembling and stabilization.

Figure 6. Effects of Ad2/HIF-1α/VP16 on endothelial cell proliferation. HPAECs maintained in EBM containing 0.5% FBS only were infected with Ad2/EV (approximately 100 MOI) or Ad2/HIF-1α/VP16 or treated with VEGF (30 ng/mL). Number of cells was counted 48 hours later (A). Separate cells were treated with the conditioned medium harvested from the donor HPAECs over a period of 24 hours (B). Data are expressed as mean±SEM (n=3). *P<0.05 compared with controls or EV.

Role of HIF-1 in Hypoxic Induction of Multiple Angiogenic Factors

In a rabbit model of hindlimb ischemia, administration of a DNA plasmid encoding HIF-1α/VP16 increases angiogenesis and blood supply. Intramyocardial injection of the plasmid DNA encoding HIF-1α/VP16 also enhances myocardial angiogenesis after infarction. Transgenic expression of a constitutively active HIF-1α mutant results in hypervascularity. In this study, direct infection of endothelial cells with Ad2/HIF-1α/VP16 or conditioned medium harvested from donor endothelial cells expressing HIF-1α/VP16 promoted endothelial cell proliferation and tube formation, suggesting that activation of endothelial cells by HIF-1α/VP16 was mainly attributable to secreted angiogenic proteins. Furthermore, hypoxic induction of VEGF, Ang-2, and Ang-4 was mimicked by HIF-1α/VP16 or desferrioxamine, which inhibits the prolyl hydroxylases and thus stabilizes endogenous HIF-1α, and abolished by adenovirus-mediated expression of a dominant-negative form of HIF-1α. These studies are the first to suggest that HIF-1 mediates the hypoxic upregulation of Ang-2 and Ang-4. HIF-1α may serve as a critical mediator of the angiogenic response to hypoxia by inducing VEGF and angiopoietins, which are essential for the formation of morphologically and functionally normal vessels. Desferrioxamine and HIF-1α/VP16 also mimicked the hypoxic upregulation of PDGF and PIGF mRNA levels in HCAECs but not in HPAECs. Although the precise role of HIF-1 in the hypoxic induction of PDGF and PIGF remains to be determined, these results suggest their participation in the angiogenic response to hypoxia. Recent studies suggest that PDGF and FGF-2 synergistically induce vascular network, which remains stable for more than a year even after depletion of angiogenic factors, and together markedly stimulate arteriogenesis. PIGF and VEGF also synergistically induce angiogenesis, whereas PIGF alone enhances arteriogenesis through selective activation of VEGFR1. Formation of normal vessels likely requires the orchestration of a variety of endothelial growth factors, their receptors, and matrix proteins, which play different roles at different stages of the multiple step angiogenic process.
Contribution of Angiopoietin/Tie-2 System to the Angiogenic Response to Hypoxia

VEGF and angiopoietins play separate but essential roles at different stages of vessel formation. VEGF activates the endothelial cells and prepares the environment at the initial stage of vascular development, whereas Ang-1 is crucial in the subsequent assembly of newly formed vasculature and in the maintenance of vascular integrity. Transgenic overexpression of VEGF alone in mice results in increased number of vascular vessels with edema, inflammation, or vascular leakage. In contrast, transgenic mice overexpressing a combination of VEGF and Ang-1 results in increased number of vascular vessels with edema, inflammation, or vascular leakage. In contrast, transgenic mice overexpressing a combination of VEGF and Ang-1 results in increased number of vascular vessels with edema, inflammation, or vascular leakage. In contrast, transgenic mice overexpressing a combination of VEGF and Ang-1 results in increased number of vascular vessels with edema, inflammation, or vascular leakage. In contrast, transgenic mice overexpressing a combination of VEGF and Ang-1 results in increased number of vascular vessels with edema, inflammation, or vascular leakage. In contrast, transgenic mice overexpressing a combination of VEGF and Ang-1 results in increased number of vascular vessels with edema, inflammation, or vascular leakage.

Figure 7. Effects of Ad2/HIF-1α/VP16 on endothelial tube formation. Noninfected HPAECs seeded on Matrigel were cultured for 8 hours in conditioned medium that was harvested from donor HPAECs (A) maintained in EBM containing 2% FBS (control, C) or (B) infected with Ad2/CMVVEV (EV) or (C) Ad2/HIF-1α/VP16 (approximately 30 MOI; HIF1α). Additional HPAECs were maintained in the conditioned medium from the donor cells infected with Ad2/HIF-1α/VP16 in the presence of either Tie-2Fc (20 μg/mL, HIF1α+Fc) (D) or VEGFR2 kinase inhibitor I (1 μmol/L, HIF1α+VEGFRI) (E). F, Length of tube-like structures was counted (mean±SEM, n=3). *P<0.05 compared with controls or EV; †P<0.05 compared with HIF1α alone.

In summary, our studies suggest that HIF-1 may serve as a global mediator of the angiogenic response to hypoxia in cultured human endothelial cells by inducing multiple angiogenic factors including VEGF and angiopoietins. Ang-4 may substitute for Ang-1 to participate in HIF-mediated angiogenic response to hypoxia. Activation of the angiopoietin/Tie-2 system by HIF-1 may help explain the different nature of hypervascularity induced by HIF-1α and VEGF.

Acknowledgments

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(20 μg/mL) for 6 hours. D. Length of the tube-like structures was counted (mean±SEM, n=3). *P<0.05 compared with controls; †P<0.05 compared with hypoxia alone.

References


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Online Methods and Materials

Detection of endothelial cell apoptosis by Hoechst 33258 staining

HPAECs were seeded on 4-well chamber slides and cultured in optimal growth medium (EBM supplemented with 2% FBS and SingleQuots). Confluent cells were then maintained with optimal growth medium, EBM alone (without FBS and growth factors), or EBM plus recombinant angiopoietin-4 for 24 hours. Apoptosis was detected using Hoechst 33258 staining, as described previously.\(^1\) Briefly, cells were fixed and stained simultaneously using a staining solution containing 4% formaldehyde, 0.6% Nonidet P-40, and 18.7 µM Hoechst 33258 (Sigma) in PBS at room temperature.
References


Figure Legends

**Online Figure 1. Effects of hypoxia or Ad2/HIF-1α/VP16 on the mRNA levels of Ang-4 in human fetal cardiac cells.** Human fetal cardiac cells were maintained under normoxic conditions, subjected to hypoxia, or infected with Ad2/EV (100 MOI) or Ad2/HIF-1α/VP16 (100 MOI). The mRNA levels were measured by TaqMan analysis and normalized to 18S RNA (mean±SEM, n≥3). The experiments were repeated multiple times and similar results were obtained. * and † indicate p<0.05 and 0.01, respectively, compared to normoxic controls.

**Online Figure 2. Effects of Ang-4 on endothelial cell migration.** The assay was performed as described previously.² Wounded human pulmonary artery endothelial cell monolayers were maintained in EBM containing 4% FBS (EBM), optimal growth medium (GM) containing a cocktail of growth factors and FBS, or EBM plus Ang-4 (0.3 and 1 µg/ml) for 12 hours. The recovered surface areas were then measured. Data are expressed as mm²/cm² recovered area (mean±SEM, n=3). * indicates p<0.05 compared to EBM.
Online Table 1. Primers and probes for TaqMan 5' nuclease fluorogenic quantitative PCR analysis. The primers and probes for the measurement of the mRNA levels of human Ang-1, Ang-2, Ang-4, PDGF, PIGF, VEGF, and 18S rRNA were designed according to ABI-Perkin Elmer guidelines and synthesized by Operon.

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Online Figure 1

Online Figure 2