Cell Type–Specific Regulation of Angiogenic Growth Factor Gene Expression and Induction of Angiogenesis in Nonischemic Tissue by a Constitutively Active Form of Hypoxia-Inducible Factor 1

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Abstract—Understanding molecular mechanisms regulating angiogenesis may lead to novel therapies for ischemic disorders. Hypoxia-inducible factor 1 (HIF-1) activates vascular endothelial growth factor (VEGF) gene expression in hypoxic/ischemic tissue. In this study we demonstrate that exposure of primary cultures of cardiac and vascular cells to hypoxia or AdCA5, an adenovirus encoding a constitutively active form of HIF-1α, modulates the expression of genes encoding the angiogenic factors angiopoietin-1 (ANGPT1), ANGPT2, placental growth factor, and platelet-derived growth factor-B. Loss-of-function effects were also observed in HIF-1α-null embryonic stem cells. Depending on the cell type, expression of ANGPT1 and ANGPT2 was either activated or repressed in response to hypoxia or AdCA5. In all cases, there was complete concordance between the effects of hypoxia and AdCA5. Injection of AdCA5 into mouse eyes induced neovascularization in multiple capillary beds, including those not responsive to VEGF alone. Analysis of gene expression revealed increased expression of ANGPT1, ANGPT2, platelet-derived growth factor-B, placental growth factor, and VEGF mRNA in AdCA5-injected eyes. These results indicate that HIF-1 functions as a master regulator of angiogenesis by controlling the expression of multiple angiogenic growth factors and that adenovirus-mediated expression of a constitutively active form of HIF-1α is sufficient to induce angiogenesis in nonischemic tissue of an adult animal. (Circ Res. 2003;93:1074-1081.)

Key Words: angiogenesis gene therapy hypoxia

Ischemic heart disease is a major cause of mortality that is treated by pharmacologic agents, balloon angioplasty, and coronary artery bypass graft surgery. Therapeutic angiogenesis aims to stimulate neovascularization by delivery of an angiogenic factor or its protein-coding DNA sequence. Phase I clinical trials have demonstrated safety, but phase II trials have not demonstrated efficacy. This outcome reflects the complexity of angiogenesis, which involves the temporally and spatially orchestrated expression by multiple cell types of multiple angiogenic factors, including vascular endothelial growth factor (VEGF), angiopoietin-1 (ANGPT1), ANGPT2, fibroblast growth factor 2 (FGF2), placental growth factor (PLGF), platelet-derived growth factor-B (PDGFB), transforming growth factor-β, and others. In ischemic tissue, angiogenic factors are produced by a variety of cell types, including myocytes, fibroblasts, and vascular cells.

VEGF is produced early in the angiogenic cascade and is responsible for initial activation of endothelial cells (ECs). Transgenic expression of VEGF in mouse skin results in increased numbers of blood vessels that manifest excessive permeability, whereas expression of both VEGF and ANGPT1 results in increased vessels without excessive permeability. However, transgenic coexpression of ANGPT1 in the heart blocks the angiogenic effect of VEGF. PLGF plays a critical role in ischemia-induced angiogenesis and has synergistic effects with VEGF in some tissues. Synergistic effects of combined treatment with PDGF-BB and FGF2 have also been reported. These data suggest that increased expression of a single angiogenic factor is not sufficient for functional vascularization and that effects of angiogenic factors are tissue specific.

Hypoxia-inducible factor 1 (HIF-1) is a transcriptional activator that functions as a global regulator of oxygen homeostasis. HIF-1 is a heterodimer consisting of a constitutively expressed HIF-1α subunit and an O2-regulated
HIF-1α subunit. Hydroxylation of proline residues 402 and 564 and asparagine residue 803 in HIF-1α regulates protein stability and transactivation function in an O2-dependent manner. Homozygosity for a null allele at the mouse Hif1α locus encoding HIF-1α results in embryonic lethality attributable to failed vascularization. More than 60 HIF-1 target genes have been identified, including VEGF. Transgenic expression of HIF-1α in mouse skin results in increased vascularization without excessive permeability, suggesting that HIF-1 may regulate angiogenic factors in addition to VEGF, leading to a more physiological outcome than that resulting from VEGF expression alone, but definitive results supporting this hypothesis have not been reported. In this study, we demonstrate that HIF-1 regulates the expression of multiple critical angiogenic growth factors in a cell type-specific manner and that administration of an adenovirus expressing a constitutively active form of HIF-1α is sufficient to induce angiogenesis in nonischemic tissue.

Materials and Methods

AdCA5 Construction

The nucleotide sequence encoding HIF-1αCA5 was excised from pCEP4/HIF-1α(1-391/521-826) and ligated into pAdTrack-CMV. Homologous recombination was performed in Escherichia coli between the shuttle vector pAdTrack-CMV and adenoviral plasmid pAdEasy-1. Recombinant plasmid was transfected into 293 cells for adenovirus production. Missense mutations (Pro567Thr and Pro658Gln) were introduced by polymerase chain reaction (PCR) during construction of pCEP4/HIF-1α(1-391/521-826) and are required for constitutive activity of CA5. Large-scale adenoviral production was performed at the NHLBI PEGT Vector Core Facility, University of Pittsburgh.

Cell Culture

Ventricular cardiac myocytes and fibroblasts were isolated from neonatal rat hearts. Cells were dispersed by incubation at 37°C in HEPES-buffered salt solution containing 2 mg/mL trypsin and 20 μg/mL deoxyribonuclease I. Dispersed cells were preplated for 45 minutes, and attached cells were maintained to establish the fibroblast culture. Unattached cells were replated on 0.1% gelatin–coated dishes, cultured in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin for 24 hours, and then maintained in DMEM with 1% FCS (cardiac myocyte culture). The medium contained 100 μmol/L bromodeoxyuridine for the first 48 hours. Pulmonary artery endothelial cells were cultured in EBM-2 medium, supplemented with EGM-2 SingleQuots (Clonetics) and FBS at a final concentration of 10%. Smooth muscle cells (SMCs) were isolated from the pulmonary arteries of 150-g Sprague-Dawley rats and cultured in Ham’s F-12 medium (Invitrogen) supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were subjected to hypoxia by placing tissue culture dishes in a modular incubator chamber (Billups-Rothenberg, Inc) which was flushed with a gas mixture consisting of 1% O2, 5% CO2, and balance N2. Cells subjected to adenosine vascular injury were inoculated with 25 plaque-forming units (pfu) per cell and incubated for 24 hours under standard tissue culture conditions (95% air and 5% CO2).

RNA Isolation

Mice were euthanized, and eyes were homogenized individually in 0.4 mL of a solution containing 4 mol/L guanidinium thiocyanate, 25 mmol/L sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 mol/L 2-mercaptoethanol, extracted with phenol-chloroform-isooamyl alcohol, and total RNA was precipitated by addition of isopropanol as described. RNA was isolated from tissue culture cells using Trizol (Invitrogen).

Reverse Transcriptase–Polymerase Chain Reaction Assays

Aliquots of 5 μg of total RNA were reverse-transcribed to cDNA using Superscript First-Strand Synthesis System for reverse transcriptase (RT)-PCR kit with oligo-dT and random hexamer primers (Invitrogen). For each primer pair (oligonucleotide sequences available on request), PCR was optimized for Mg2+ concentration and cycle number to obtain linearity between the amount of input RT product (1, 2, and 4 μL) and output PCR product. For HIF-1α, the primer sequences chosen are identical in the human and mouse genes and are present in HIF-1αCA5.

Real-Time RT-PCR Assays

RNA was DNase treated and purified using RNeasy (Qiagen). First-strand synthesis was performed with iScript cDNA Synthesis system (BioRad) and 5 μg of RNA. cDNA was diluted 1:10, and 2 μL was added to each PCR. Primers were designed using Beacon Designer software (sequences available on request). Real-Time PCR was performed using iQ SYBR Green Supermix and the iCycler Real-Time PCR Detection System (BioRad). Expression of each angiogenic growth factor (target) mRNA was calculated based on the threshold cycle (Ct) as 2^{-ΔΔCt}, where ΔCt = Ct,Target–Ct,18S and Δ(ΔCt) = ΔCt,1%–ΔCt,20% (for embryonic stem [ES] cells) or ΔCt,AdCA5–ΔCt,AdCA5̂ (for tissue samples).

Ocular Injection of Adenoviral Vectors

Six- to 8-week-old C57BL/6J male mice (National Cancer Institute, Frederick, Md.) were injected with 1 μL containing 1.2 × 1010 particles (~2.4 × 1010 pfu) of AdCA5 into the right eye using a microinjection pump (Harvard Apparatus) and pulled glass micropipet. AdLaCZ was injected into the left eye. Mice were anesthetized by intramuscular injection of ketamine and xylazine, the eyes were dilated, and the needle was passed through the sclera just behind the limbus as visualized with a dissecting microscope. The needle tip was positioned either within the vitreous cavity or subretinal space before injection. Experimental procedures were performed according to protocols approved by The Johns Hopkins University Animal Care and Use Committee.

Immunohistochemistry

Eyes from mice euthanized 1 to 6 days after injection were embedded in optimal tissue cutting medium (Sakura Finetek) and frozen. Sections of 10 μm were fixed for 30 minutes with cold 4% paraformaldehyde in PBS and incubated with cold methanol/H2O2 for 10 minutes. To visualize the retinal vasculature of eyes after intravitreal injection, sections were preincubated for 30 minutes with 10% normal swine serum in Tris-buffered saline (TBS) and then incubated with a 1:20 dilution of biotinylated Griffonia simplicifolia isoelectin B4 (Vector Laboratories) for 2 hours. Slides were rinsed with TBS, incubated for 1 hour with a 1:100 dilution of avidin–conjugated horseradish peroxidase (Vector Laboratories), rinsed with 0.05 mol/L Tris (pH 7.6), incubated with Stabable DAB (Régen) for 3 minutes, rinsed with water, counterstained with eosin, dehydrated through 70%, 95%, and 100% ethanol. Coverslips were mounted with Cytoseal (Richard Allen Scientific). For smooth muscle actin (SMA) staining, slides were blocked with 10% normal rabbit serum (NRS) in TBS and incubated with a 1:50 dilution of anti-SMA monoclonal antibody (BioGenex, San Ramon, Calif) in 2% NRS/TBS overnight at 4°C. The slides were rinsed with 2% NRS/TBS, incubated with 1:25 rabbit anti-mouse IgG (Arnel, Brooklyn, NY) in 2% NRS/TBS for 45 minutes, rinsed with 2% NRS/TBS, and incubated with 1:400 mouse peroxidase anti-peroxidase (Arnel) in 2% NRS/TBS for 45 minutes. The slides were rinsed with 0.05 mol/L Tris pH 7.6, reacted with Stabable DAB for 3 minutes, rinsed with water, counterstained with eosin, dehydrated, and mounted.

For eyes that received subretinal injections, the sections were preincubated for 30 minutes at room temperature with 10% normal...
swine serum in TBS, incubated with a 1:40 dilution of biotinylated *Griffonia simplicifolia* isoelectin B4 in 1% normal swine serum at 4°C overnight, and rinsed with TBS containing 0.1% Triton X-100 and then with TBS. The slides were incubated for 1 hour with streptavidin-phosphatase and rinsed as above, and the reaction product was visualized using HistoMark Red kit (KPL). After rinsing with water, the slides were counterstained with hematoxylin, dehydrated, and mounted. To detect PLGF expression, sections were preincubated with a blocking solution of 1% skim milk in TBS for 30 minutes and incubated overnight with a 1:200 dilution of biotin-conjugated bovine anti-goat antibody in blocking solution for 1 hour, rinsed, and incubated with streptavidin-phosphatase, and staining was visualized with HistoMark Red. HIF-1α expression was detected using mouse monoclonal antibody clone 54 (BD Biosciences).

**Results**

**Regulation of Angiogenic Growth Factor Gene Expression by HIF-1**

To test the hypothesis that HIF-1 coordinately regulates the expression of multiple genes encoding angiogenic growth factors, we analyzed gene expression in primary cultures of cardiac myocytes and fibroblasts as well as arterial ECs and SMCs. Neovascular sprouting requires EC activation mediated by binding of VEGF to its receptors (VEGFRs) as well as alterations of EC-SMC interactions mediated by binding of ANGPT1 and ANGPT2 to the TIE2 receptor on ECs.7,26 However, the roles of ANGPT1 and ANGPT2 are poorly understood and seem to be cell-type specific.5 PDGFB also regulates EC-SMC interactions.27 PLGF binds to VEGFR1 and is required for ischemia-induced angiogenesis.9 In hypoxic cardiac myocytes, VEGF and PLGF mRNA levels increased, ANGPT1 mRNA levels decreased, and expression of ANGPT2 and PDGFB mRNA was unchanged (Figure 1A). Cardiac fibroblasts showed similar changes in gene expression, except that ANGPT1 mRNA levels were not modulated by hypoxia (Figure 1B). In ECs, mRNAs encoding VEGF, ANGPT2, PLGF, and PDGFB were induced by hypoxia.

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**Figure 1.** Analysis of angiogenic factor gene expression in cultured cells. Primary cultures of cardiac myocytes (A), cardiac fibroblasts (B), arterial endothelial cells (C), or arterial SMCs (D) were incubated for 24 hours under nonhypoxic (20% O2) or hypoxic (1% O2) conditions or under nonhypoxic conditions in the presence of AdLacZ or AdCA5 at a multiplicity of infection of 25 pfu/cell. Total RNA was analyzed by RT-PCR. E, Structure of HIF-1α and CA5. The basic helix-loop-helix (bHLH) and PAS domains of HIF-1α that are required for dimerization with HIF-1β and DNA binding are intact in CA5, as are the amino- and carboxyl-terminal transactivation domains (TAD-N and TAD-C, respectively). However, O2-dependent degradation of HIF-1α under hypoxic conditions is impaired by deletion of amino acids 392 through 520 and the missense mutations Pro567Thr (P567Q) and Pro658Gln (P658Q). F, Summary of changes in gene expression induced by hypoxia or AdCA5. Exposure of cardiac myocytes (CM), cardiac fibroblasts (CF), arterial ECs (EC), and arterial SMCs (SM) to 1% O2 or AdCA5 infection either increased (green), decreased (red), or had no effect (yellow) on the expression of the indicated angiogenic growth factor mRNAs relative to the appropriate control (20% O2 and AdLacZ infection, respectively). G, Gene expression in ES cells. Real-time RT-PCR was performed to analyze total RNA isolated from *Hif1a*+/- and *Hif1a*−/− ES cells that were incubated under nonhypoxic (20% O2) or hypoxic (1% O2) conditions for 24 hours. The mean (n=3) fold induction in hypoxic cells is shown for each mRNA.
in Hif1α−/− cells, whereas PDGFB and ANGPT2 expression was not induced by hypoxia in wild-type or HIF-1α-null ES cells (Figure 1G). Taken together, the results in Figure 1 demonstrate that the expression of VEGF, PLGF, ANGPT1, ANGPT2, and PDGFB mRNA is induced by hypoxia in a HIF-1α-dependent manner in at least one of the five cell types studied. Furthermore, in different cell types, expression of ANGPT1 and ANGPT2 mRNA can be either induced or repressed by hypoxia in a HIF-1α-dependent manner.

**Angiogenesis Induced by Subretinal Injection of AdCA5**

To determine whether increased HIF-1 activity is sufficient to induce angiogenesis in nonischemic tissue in vivo, we analyzed the effect of AdCA5 on vascularization of the retina, which is perfused by superficial and deep capillary beds that are located within the ganglion cell layer and inner nuclear layer (INL), respectively. AdCA5 and AdLacZ were administered to the right and left eyes, respectively, of C57BL/6J mice by subretinal injection (Figure 2). Seventy-two hours later, the eyes were harvested and sections were stained with *Griffonia simplicifolia* lectin (GSA), which selectively binds to vascular endothelial cells,28 or with antibodies against α-SMA, a marker for pericytes and vascular SMCs. In the retina of AdCA5-treated eyes, a marked increase in GSA and SMA staining was observed in the deep capillary bed of the INL, indicating vascular proliferation (Figure 2, large arrows). Staining was also detected at the site of subretinal injection (Figure 2, small arrows), indicating neovascularization, because vessels are not normally found in this region.

Immunohistochemical analysis of eyes harvested 24 hours after injection revealed expression of HIF-1α protein at the site of AdCA5 injection (Figure 3, top). mRNA expression in eyes injected with AdCA5 and AdLacZ was determined by real-time RT-PCR. HIF-1α mRNA levels were significantly increased in AdCA5-treated eyes 24 hours after injection (Figure 4). PLGF mRNA expression was also markedly increased, whereas no significant difference in the expression of VEGF, ANGPT1, ANGPT2, or PDGFB mRNA relative to the contralateral eye was observed. Increased PLGF protein expression was detected in the retinas of eyes injected with AdCA5 as late as 6 days after injection (Figure 3, bottom).

**Angiogenesis Induced by Intravitreous Injection of AdCA5**

We also performed intravitreous injection of AdCA5 and AdLacZ (Figure 5). Normally, no vascular cells extend above the internal limiting membrane (ILM) of the retina, and whenever this occurs it indicates neovascularization. In eyes that received an intravitreous injection of AdCA5, clusters of GSA- and SMA-positive cells (arrows in Figure 5, left) extended above the ILM (arrowheads) into the vitreous cavity. In contrast, eyes injected with AdLacZ showed normal staining patterns for GSA and SMA confined within the ILM, indicating normal retinal vessels and no neovascularization. Intravitreous injection of AdCA5 also resulted in numerous GSA- and SMA-positive cells within the cornea (arrows in Figure 5, right) and in the anterior chamber angle (arrowheads), indicating severe corneal and iris neovascular-
intravitreal injection of AdCA5 (right eye) or AdLacZ (left eye). Primers that amplify both mouse and human sequences were used to measure total HIF-1α (mouse HIF-1α+CA5) expression. In AdCA5-injected eyes overexpressing HIF-1α, increased PLGF and, to a lesser extent, VEGF, ANGPT1, ANGPT2, and PDGFB mRNA expression was detected, with expression peaking at 48 hours after injection (Figure 6). The expression levels showed considerable variability between mice that reflects variability in transduction efficiency attributable to technical difficulties associated with intravitreal injections in mice, including leakage of injectate through the scleral puncture site. However, the AdCA5-injected eye with the highest level of HIF-1α mRNA also expressed the highest level of PLGF, VEGF, ANGPT1, and ANGPT2 mRNA relative to the contralateral AdLacZ-injected eye. Significantly, in AdCA5-injected eyes that showed no increase in HIF-1α mRNA expression relative to the contralateral eye (indicating injection failure), no increase in expression of mRNAs encoding angiogenic growth factors was observed (data not shown).

**Discussion**

The data presented above support three major conclusions. First, both loss-of-function studies in ES cells and gain-of-function in primary cardiac and vascular cells indicate that HIF-1 plays a major role in angiogenesis induced by hypoxia/ischemia by coordinately regulating the expression of genes encoding multiple critical angiogenic growth factors (VEGF, PLGF, ANGPT1, ANGPT2, and PDGFB) in a cell type–specific manner (Figure 7). This result may provide a mechanistic basis for the observation that transgenic mice expressing a constitutively active form of HIF-1α in the skin manifest increased vascularization without the increased vascular permeability observed in transgenic mice expressing VEGF from the same promoter. However, additional studies are required to determine which of the HIF-1–regulated angiogenic factors other than VEGF is overexpressed in keratinocytes of these mice. Because cis–acting hypoxia-responsive elements containing HIF-1 binding sites have not been demonstrated for the genes encoding angiogenic factors identified in this study (other than VEGF), it remains to be determined whether HIF-1–dependent regulation of these genes is direct or indirect. However, whether by direct or indirect mechanisms, the data indicate that HIF-1 is a pleiotropic mediator of the angiogenic program.

Second, we have demonstrated for the first time that HIF-1 can function as either an activator or repressor of ANGPT1 and ANGPT2 gene expression in a cell type–specific manner. The molecular mechanism underlying this surprising observation remains to be established. These results are consistent with published data, indicating that these factors can either induce or inhibit angiogenesis, depending on the cellular context. The remarkable cell type–specific regulation of gene expression that is demonstrated in this study underscores the general principle that HIF-1 functions as a messenger to the nucleus, signaling hypoxia. The response to this signal is dependent on prior programming of the cell, ie, the presence or absence of other transcriptional regulators of potential HIF-1 target genes.
Third, we have demonstrated for the first time that adenoviral vector–mediated intraocular expression of a constitutively active form of HIF-1α causes several types of neovascularization in the absence of hypoxia or ischemia. GSA and SMA staining demonstrated that these vessels contain both ECs and SMCs/pericytes. A link between HIF-1 expression and VEGF expression in ischemic retina has been well-established,29 as has a role for VEGF in ischemia-induced retinal neovascularization.30,31 However, the ability of AdCA5 to induce sprouting of new vessels that extend into the vitreous cavity from the superficial capillary bed, as occurs in patients with diabetic retinopathy, contrasts with the inability of high levels of VEGF alone to do so. In primates, multiple intravitreous injections of VEGF32 or implantation of a pellet that provides sustained intraocular release of VEGF33 caused many changes to retinal vessels, including dilation, leakage, and microaneurysms, but failed to cause any retinal neovascularization. Sustained doxycycline-inducible, retina-specific expression of VEGF165 in adult transgenic mice caused neovascularization from the deep capillary bed but not from superficial capillaries.34 These results indicate that increased levels of a single VEGF isoform are not sufficient to cause new vessels to sprout from the superficial vessels. Our data suggest a prominent role for PLGF in the neovascularization induced by AdCA5. Previous studies have demonstrated that PLGF can act synergistically with VEGF to stimulate neovascularization and that PLGF is required for neovascularization in the ischemic retina.9,10 Thus, the combined effect of increased PLGF and VEGF expression may underlie neovascularization in the superficial capillary bed induced by intravitreous injection of AdCA5. The failure to demonstrate increased expression of mRNAs encoding angiogenic growth factors other than PLGF after subretinal injection may be attributable to the fact that gene expression was assayed only at 24 hours after subretinal injection. Our more extensive time course after intravitreous injection of AdCA5 demonstrated increased expression of PLGF, VEGF, ANGPT1, ANGPT2, and PDGFB mRNA that peaked at 48 hours.

Two other constitutively active forms of HIF-1α expressed by either transgenic20 or gene therapy35,36 approaches have been shown to increase vascularization during development or in response to ischemia, respectively. Cardiomyocyte-specific expression in transgenic mice of PR39, a peptide that selectively inhibits degradation of HIF-1α, was also associated with increased vascularization and protection against ischemia.37 Our data suggest potential molecular mechanisms underlying the angiogenic effects observed in these prior studies. The coordinate activation of VEGF and PLGF and downregulation of ANGPT1 mRNA expression by HIF-1 in cardiomyocytes is an interesting finding, because recent studies suggest that these changes in angiogenic factor expression may stimulate both angiogenesis and arteriogenesis/collateralization in the ischemic heart.8,10,38,39 The dem-

**Figure 5.** Histochemical analysis of retinal vasculature after intravitreous injection. Sections from the indicated regions of mouse eyes that received an intravitreous injection of AdCA5 or AdLacZ 6 days earlier were stained with GSA lectin or anti-SMA antibodies. Left (red box), In sections from AdCA5–injected eyes, retinal neovascular tufts (arrows) were seen projecting above the inner limiting membrane (ILM; demarcated by arrowheads). Right (green box), Corneal neovascularization that stained positively for GSA and SMA (arrows) was observed in AdCA5–injected eyes but not in AdLacZ–injected eyes. There was also staining for GSA and SMA in the irido-corneal angle (arrowheads).
onstration that neovascularization is rapidly induced in AdCA5-injected eyes in the absence of ischemia is noteworthy, because most patients with coronary artery disease do not have ischemia at rest. Additional studies are required to determine whether administration of AdCA5, other modified forms of HIF-1α,20,36 or peptides that inhibit HIF-1α ubiquitination or degradation 37,40 may be of therapeutic utility in patients with ischemic cardiovascular disease.

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