Angiogenesis Is Induced in a Rabbit Model of Hindlimb Ischemia by Naked DNA Encoding an HIF-1α/VP16 Hybrid Transcription Factor

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Background—Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor that regulates expression of genes involved in O₂ homeostasis, including vascular endothelial growth factor (VEGF), a potent stimulator of angiogenesis. We sought to exploit this native adaptive response to hypoxia as a treatment for chronic ischemia.

Methods and Results—A hybrid protein consisting of DNA-binding and dimerization domains from the HIF-1α subunit and the transactivation domain from herpes simplex virus VP16 protein was constructed to create a strong, constitutive transcriptional activator. After transfection into HeLa, C6, and Hep3B cells, this chimeric transcription factor was shown to activate expression of the endogenous VEGF gene, as well as several other HIF-1 target genes in vitro. The bioactivity of HIF-1α/VP16 hybrid gene transfer in vivo was examined in a rabbit model of hindlimb ischemia. Administration of HIF-1α/VP16 was associated with significant improvements in calf blood pressure ratio, angiographic score, resting and maximal regional blood flow, and capillary density (all P<0.01).

Conclusions—The HIF-1α/VP16 hybrid transcription factor is able to promote significant improvement in perfusion of an ischemic limb. These results confirm the feasibility of a novel approach for therapeutic angiogenesis in which neovascularization may be achieved indirectly by use of a transcriptional regulatory strategy. (Circulation. 2000;102:2255-2261.)

Key Words: transcription factors • ischemia • angiogenesis • collateral circulation • growth substances

Hypoxia-inducible factor-1 (HIF-1) was first identified as a factor critical for the inducible activity of the erythropoietin (EPO) 3' enhancer and is now recognized to be a key regulator of gene expression in response to changes in cellular oxygen tension. As a heterodimeric transcription factor, HIF-1 is composed of 2 basic helix-loop-helix (bHLH)-Per-ARNT-Sim (PAS) proteins, HIF-1α and HIF-1β. The HIF-1α subunit is an 826-amino-acid protein that is unique to HIF-1, whereas HIF-1β is the previously identified aryl hydrocarbon nuclear translocator (ARNT) protein. The bHLH and PAS domains of HIF-1α and HIF-1β comprise the amino-terminal half of each protein and are required for DNA binding and dimerization, whereas the transactivation domains are located downstream. Two distinct transactivation domains have been identified in HIF-1α; one is located in the middle of the protein (amino acids [aa] 530 to 580), and the other is found at the extreme carboxy-terminal (aa 780 to 826).

Both HIF-α and HIF-1β mRNAs are expressed in most tissues of humans, mouse, and rat. There is conflicting evidence as to whether steady-state levels of HIF-1α mRNA may be augmented slightly or not at all in response to a hypoxic stimulus. However, HIF-1α protein levels and HIF-1 DNA-binding activity both increase markedly as cellular oxygen concentration is reduced. When cells maintained at low oxygen tension are returned to a nonhypoxic environment, HIF-1α protein levels decay rapidly as the protein is degraded via the ubiquitin-proteosome pathway. This observation has suggested that stabilization of the HIF-1α protein is one mechanism for hypoxic induction of HIF-1α protein expression and consequently HIF-1 activity. The HIF-1α protein domain responsible for hypoxia-dependent stabilization has been localized to the central region of the protein between amino acids 400 and 600.

We sought to exploit the adaptive response to hypoxia as an alternative approach for the treatment of tissue ischemia. Vascular endothelial growth factor (VEGF), an endothelial cell–specific mitogen and potent stimulator of angiogenesis, is a target of HIF-1α-mediated transcriptional activation. Previous studies performed in animal models of peripheral and myocardial ischemia have indicated that administration...
of VEGF as a recombinant protein, as naked plasmid DNA, or as a recombinant adenovirus may augment vascularity in ischemic tissues. Given these findings, we considered that a modified HIF-1α transcription factor administered via gene transfer might induce expression of VEGF and/or downstream targets, ultimately leading to therapeutic neovascularization of ischemic tissues.

Accordingly, we constructed a constitutively active form of HIF-1α consisting of the DNA-binding and dimerization domains from HIF-1α and the transactivation domain from herpes simplex virus VP16 protein. In vitro analyses of the HIF-1α/VP16 hybrid transcription factor demonstrated that upregulation of endogenous VEGF gene expression in HeLa and C6 cells as well as both VEGF and EPO in Hep3B cells was independent of hypoxia. Similar results were documented for other HIF-1 target genes. Experiments were then performed in a rabbit hindlimb ischemia model to test the hypothesis that transfection with naked plasmid DNA encoding HIF-1α/VP16 could enhance collateral vessel development. Results of these studies suggest that intramuscular injection of naked DNA encoding HIF-1α/VP16 may represent a viable treatment strategy for tissue ischemia.

### Methods

**Recombinant Plasmids**

The full-length (aa 1 to 820) HIF-1α gene was isolated by polymerase chain reaction (PCR) using cDNA PCR Kit (Clontech) from a HeLa cell cDNA library (Clontech) and inserted between the KpnI and XbaI sites of the expression vector, pcDNA3 (Invitrogen). In this plasmid, gene expression is controlled by the cytomegalovirus immediate early enhancer/promoter. The HIF-1α/VP16 hybrid was constructed by truncating HIF-1α at aa 390 (an AflII site) and then joining the transactivation domain of HSV VP-16 downstream. A VP16 fragment (aa 413 to 490) with AflII and XbaI ends was amplified by PCR using Vent polymerase (New England Biolabs), and this fragment was cloned into the appropriate sites of the pcDNA3/HIF-1α construct. The integrity of all sequences generated by PCR was verified by DNA sequencing with an Applied Biosystems 373 DNA Sequencer. Restriction enzymes and DNA-modifying enzymes were obtained from either New England Biolabs or Life Technologies, Inc and used according to the manufacturer's specifications. Plasmid DNAs were purified with kits obtained from Qiagen. The plasmid constructs pVEGF165 and pCMVβ have been described previously.

**Transient Transfections**

Hep3B cells were grown in DMEM–high glucose (Irvine Scientific) with 10% FBS (SRH Biosciences). Hep3B cells were maintained in minimum essential (Eagle's) medium supplemented with Earle's balanced salt solution and 10% FBS. For the analysis of VEGF and EPO production, HeLa, Hep3B (2 × 10^6 cells/well), and C6 (5 × 10^5 cells/well) cells were transfected in triplicate in 6-well dishes with Lipofectamine (Life Technologies); 1 μg DNA and 4 μL Lipofectamine per well for HeLa and C6, 1 μg DNA and 2.5 μL Lipofectamine per well for Hep3B cells in Opti-MEM medium (Life Technologies). The transfection was allowed to proceed for 16 hours. Five hours after the termination of transfection, 1 set of dishes was maintained under normoxic conditions and 1 set was exposed to hypoxia in a gas-controlled chamber (Espec) maintained at 1% O₂, 94% N₂, and 5% CO₂. The remaining dishes were maintained with 100 mmol/L desferrioxamine. At 24 hours after induction, the culture medium was harvested and the cells were lysed in 500 μL lysis buffer (0.5% NP-40, 1 mmol/L EDTA, 50 mmol/L Tris [pH 8.0], 120 mmol/L NaCl, 100 mmol/L PMSF, 0.1 U/mL aprotinin, 1 mmol/L Pefabloc, 5 mg/mL leupeptin). VEGF and EPO concentrations were assayed by use of human VEGF- and EPO–specific ELISA kits (R&D Systems), and the total cell protein was analyzed with the Bio-Rad protein assay. ELISA values were normalized to total cell protein.

**Protein Extraction and Immunoblotting**

Hep3B cells were transfected as described above in duplicate dishes. Twenty-four hours after termination of the transfection, 1 set of dishes was treated with 100 mmol/L desferrioxamine. Cells were harvested at 4, 8, and 24 hours after induction by lysis in 600 μL of lysis buffer (see above). A total of 30 μg of protein was loaded onto Tris–glycine polyacrylamide gels (6% for HIF-1α and 12% for HIF-1α/VP16). After transfer to PVDF membranes, the filters were incubated with antibodies to HIF-1α (1:300; Novus Biologicals) or VP16 (1:100; Santa Cruz) in 5% milk powder dissolved in TBST (10 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, and 0.05% Tween 20). The filter was developed with an enhanced chemiluminescence kit (Amersham).

**Isolation of RNA and Northern Blot Analysis**

Total RNA was isolated from transfected HeLa cells with RNAzol B (Tel-Test) according to the manufacturer's instructions. Ten micrograms of RNA was loaded in each lane of a 1% agarose–0.65% formaldehyde gel. Probes (glycolytic enzymes, transferrin, the glucose transporter Glut-1) were inserts isolated from cDNA clones obtained from the ATCC; the HIF-1α probe was a KpnI/AflII fragment from the pcDNA3HIF-1α plasmid; the β-actin probe was generated by PCR from human genomic DNA. The DNA fragments were ³²P-labeled by random-primer synthesis with a commercial kit (Stratagene).

**Intramuscular Gene Transfer**

Twenty-nine rabbits were used to study the effects of intramuscular gene therapy on hindlimb ischemia. All protocols were approved by St Elizabeth's Institutional Animal Care and Use Committee. Direct intramuscular gene transfer was performed in male New Zealand White rabbits with hindlimb ischemia at 4 different sites in the ischemic limb to administer 500 μg of pVEGF165 (n = 11), 500 μg of pVEGF165 (n = 10) as a positive control, or 500 μg of pCMVβ (n = 8) as a negative control. For each animal, 125 μg in 0.5 mL of normal saline was injected at each of 4 sites. All outcome analyses (see below) were performed by observers blinded to the treatment regimen.

**Red Blood Cell, Hematocrit, and Hemoglobin Measurements**

Blood samples were drawn from the central artery of the rabbit ear with a 23-gauge needle immediately before treatment (day 0) and...
on the day rabbits were euthanized (day 40). Red blood cells, hematocrit, and hemoglobin were measured by an automatic detector (Sysmex Alpha, Sysmex Corp).

Calf Blood Pressure Ratio
Calf blood pressure was measured in both hindlimbs with a Doppler flowmeter as described previously22 immediately before treatment (day 10) as well as 1 month after initiation of the therapy (day 40). The calf blood pressure ratio was defined for each rabbit as the ratio of the systolic pressure of the ischemic limb to that of the normal limb.

In Vivo Doppler Flow Measurement
Blood flow was quantified in vivo before selective internal iliac angiography on days 10 and 40 with a 0.018-in Doppler guidewire (Cardiometrics) as previously described.25 The Doppler-derived flow calculated in this fashion has been shown to correlate with flow measurements determined by electromagnetic flowmeters both in vitro and in vivo.25

Selective Angiography
Selective internal iliac angiography was performed as previously described.22 Quantitative angiographic analysis of collateral vessel development was performed with a grid overlay composed of 2.5-mm-diameter circles arranged in rows spaced 5 mm apart. The total number of grid intersections in the medial thigh area, as well as the total number of intersections crossed by a contrast-opacified artery, were counted in a single-blind fashion. An angiographic score was calculated for each film as the ratio of grid intersections crossed by opacified arteries divided by the total number of grid intersections in the medial thigh.

Capillary Density
Tissue sections were stained for alkaline phosphatase with an indoxyl-tetrazolium method to detect capillary endothelial cells as
previously described\(^5\) and then were counterstained with eosin. Twenty different fields from the 2 muscles were randomly selected, and the numbers of capillaries and myofibers were counted to determine capillary density (capillaries/mm\(^2\)) and the capillary-to-myocyte ratio.

Statistical Analysis
All results were expressed as mean±SEM. Statistical significance was evaluated with an unpaired Student’s \(t\) test for comparisons between 2 means and ANOVA followed by Scheffé’s procedure for \(>2\) means. A value of \(P<0.05\) was considered to denote statistical significance.

Results

Analysis of the HIF-1α/VP16 Hybrid In Vitro
A hybrid transcription factor, HIF-1α/VP16, composed of the DNA-binding and dimerization domains from HIF-1α and the transactivation domain from herpes simplex virus VP16 (Figure 1) was constructed to provide strong, constitutive activation of genes normally involved in the physiological adaptation to hypoxia. As predicted, in the HeLa, C6, and Hep3B cells transfected with the HIF-1α/VP16 hybrid construct, production of VEGF was significantly enhanced in cells maintained under normoxic conditions (Figure 2, A, B, and C), indicating that the HIF-1α/VP16 hybrid is indeed constitutively active. VEGF levels in HIF-1α/VP16-transfected cells were increased \(\approx 10\)-fold over normoxia-treated, mock-transfected HeLa cells, whereas a 3-fold increase was observed in C6 and Hep3B cells. It is noteworthy that in cells transfected with a plasmid expressing the full-length HIF-1α cDNA, VEGF levels were not enhanced over mock-transfected cells. This result seems to conflict with those reported previously\(^5,31\); however, those experiments used different, potentially more sensitive measures of HIF-1α activity (eg, activation of luciferase reporter constructs). The result described here is not due to insufficient expression at the mRNA level, because Northern analysis (Figure 4) shows that amounts of the full-length HIF-1α and HIF-1α/VP16 hybrid mRNAs are approximately equivalent in the transfected cells.

In cells under normoxic conditions, EPO levels were increased 300-fold over similarly treated mock-transfected cells, and there was a 2- to 5-fold enhancement over cells subjected to hypoxia or treated with DFO (Figure 2D). Although hypoxia slightly augments EPO expression in pHIF-1α/VP16–transfected cells, we have consistently observed a reduction with DFO treatment. A similar effect has been documented previously\(^32,33\); in those experiments, EPO expression in Hep3B cells cotreated with hypoxia and DFO was less than that in cells treated with hypoxia alone. This effect was not observed with VEGF, because VEGF expression in HIF-1α/VP16-transfected cells was enhanced by DFO in all 3 cell lines examined (Figure 2, A, B, and C).

Western analysis (Figure 3) showed that the HIF-1α/VP16 hybrid accumulates to an equal extent under normoxic conditions and after induction with DFO; these levels persist during the 24 hours of incubation with DFO (Figure 3). In contrast, as previously documented,\(^3,14,15\) the full-length HIF-1α protein is unstable with normoxia. DFO treatment results in an increase in the level of HIF-1α protein at all time points examined. In this experiment, the lack of detectable endogenous HIF-1α in the mock-transfected cells may be attributable to the time point at which the analysis was performed (24 hours); a previous study\(^3\) of HIF-1α protein levels in Hep3B cells had demonstrated a peak at 4 to 8 hours of hypoxia with reduced levels after 16 hours of treatment.

As shown in Figure 4, the HIF-1α/VP16 hybrid also activated expression of lactate dehydrogenase A, phosphoglycerate kinase 1, enolase 1, aldolase A, and the Glut-1 and transferrin genes under normoxic conditions. The Northern blot analysis data confirm that the effect of HIF-1α/VP16 on VEGF induction is more potent than that achieved by hypoxia.

In Vivo Red Blood Cell Measurements
Expression of naked plasmid DNA encoding either the HIF-1α/VP16 hybrid (pHIF-1α/VP16) or human VEGF\(_{165}\) (pVEGF\(_{165}\)) was analyzed by reverse transcription–PCR and found to persist to 14 days after administration (data not shown). There was no difference in red blood cell count (520±5 to 539±15×10\(^3\)/mL) or hemoglobin (11.3±0.1 to 11.9±0.1 g/dL) for pHIF-1α/VP16, pHIF-1α/VP16, and control groups before or after treatment. Although the hematocrit appeared to increase in pHIF-1α/VP16–treated animals.
(0.327±0.001 to 0.365±0.013), a similar increase was observed in controls (0.331±0.010 to 0.363±0.006), suggesting that this result was not due to expression of the HIF-1α/VP16 hybrid transcription factor.

Analysis of Blood Pressure and Flow
The blood pressure ratio at day 40 was significantly higher in the pHIF-1α/VP16–treated animals (0.93±0.02) (P<0.01) than in the pHVEGF165 (0.82±0.03) or pCMVβ (0.69±0.02) treatment groups (Figure 5A).

At day 40, the resting and papaverine-stimulated maximal blood flows in the pHIF-1α/VP16–transfected (41.6±3.1 and 111.2±5.7 mL/min, respectively) and pHVEGF165-transfected (42.2±3.9 and 88.7±7.4 mL/min) groups were significantly higher than that of the pCMVβ group (28.7±1.5 and
angiographic score at 40 days between the pHIF-1α/VP16–treated and control (pCMVβ)-plasmid-treated rabbits at day 40; however, the maximal flow was significantly higher (P<0.05) in the animals transfected with pHIF-1α/VP16 than in the phVEGF165-treated group.

Analysis of Collateral Vessel Development

By day 40, angiographic scores in the pHIF-1α/VP16–treated (0.61±0.01) and phVEGF165–treated (0.58±0.03) rabbits were significantly greater (P<0.01 and P<0.05, respectively) than that of the negative control group (0.51±0.05) (Figures 5D and 6). There was no statistically significant difference in angiographic score at 40 days between the pHIF-1α/VP16–treated and phVEGF165–treated groups.

Capillary densities observed in the muscles of the pHIF-1α/VP16–treated group (255±13/mm²) and the phVEGF165–treated group (210±10/mm²) were significantly higher (P<0.01) than that of the pCMVβ-treated group (150±4/mm²) (Figure 5E). In addition, the capillary density was higher (P<0.05) in the animals transfected with pHIF-1α/VP16 than in the phVEGF165–treated animals. Moreover, the capillary/muscle fiber ratios of the pHIF-1α/VP16– and phVEGF165–transfected rabbits (0.88±0.06 and 0.75±0.03, respectively) were significantly higher (P<0.05) than that of the negative control (0.58±0.03). Light-microscopic evidence of frank myonecrosis was not observed in any of the groups.

Discussion

Hypoxic induction of gene expression is thought to involve the interaction of multiple transcription factors at the promoter. HIF-1α is known to interact with other DNA-binding transcription factors such as HNF-4α; cooperation with this factor is thought to allow high-level and tissue-specific expression of the EPO gene.34 Activation of EPO gene expression in response to hypoxia also depends on interaction of HIF-1α with p300, a global transcriptional coactivator.34,35 In addition, hypoxic induction of the LDH-A gene has been shown to involve a multiprotein complex composed of HIF-1α, CREB-1/ATF-1, and p300.36 Transcriptional activation of VEGF and the other HIF-1α target genes by the HIF-1α/VP16 hybrid factor in vitro and in vivo suggests that HIF-1α/VP16 is able to interact with the accessory factors required for expression of these genes in the cell types examined (human cervical epithelia, human hepatoma, rat glioma, rabbit skeletal muscle).

Anatomic evidence of revascularization in response to administration of pHIF-1α/VP16 was observed at 2 levels. First, histological examination documented an increase in vascularity at the capillary level that exceeded the negative control for both pHIF-1α/VP16 and pHVEGF165, the observed increase was greater in the HIF-1α/VP16–treated animals than in those that received phVEGF165. This increased capillary vascularity most likely contributed to the higher level of maximal blood flow in animals that received pHIF-1α/VP16 compared with the phVEGF165–treated group. Second, systematic quantification of angiographic recordings established that the number of angiographically visible collateral arteries in the HIF-1α/VP16–treated animals was similar to that achieved with phVEGF165, and both exceeded that of the negative control.

No change in red blood cell count, hematocrit, or hemoglobin was observed before or after intramuscular administration of HIF-1α/VP16. This result implies that EPO gene expression (normally limited to the fetal liver and adult kidney) was not activated, as expected if transgene expression was limited to the site of administration. This result also implies that the HIF-1α/VP16 hybrid factor does not circumvent the regulatory mechanisms maintaining tissue specificity of EPO gene expression.

Certain features of HIF-1α/VP16 make it an attractive candidate for strategies of therapeutic angiogenesis. First, it is possible that after administration of HIF-1α/VP16, expression of all isoforms of VEGF-1α may be augmented, quantitatively or qualitatively exceeding the angiogenic effect achieved by gene transfer of a single isoform.

Second, in addition to VEGF, HIF-1α/VP16 may activate expression of additional genes that promote angiogenesis. It has been reported that hypoxic induction of expression of Flt-1, 1 of 2 VEGF receptors, is mediated by a HIF-1α-binding site found upstream of the gene.37 HIF-1α may also upregulate expression of the urokinase receptor to enhance cellular migration and invasion.38 Furthermore, there may be additional, as yet uncharacterized factors involved in angiogenesis. 
that are regulated by HIF-1 and therefore possibly activated by HIF-1α/VP16.

Acknowledgments

This work was supported in part by a research fellowship from Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan (K.-G.S.), and by a research fellowship from the Interuniversity Cardiology Institute of the Netherlands (R.T.). The authors thank David Souza for sequencing the HIF-1α cDNA and Hsienwie Lu for technical assistance. We acknowledge Gregg Semenza for helpful discussions.

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Circulation. 2000;102:2255-2261
doi: 10.1161/01.CIR.102.18.2255

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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