Intramyocardial injection of naked DNA encoding HIF-1α/VP16 hybrid to enhance angiogenesis in an acute myocardial infarction model in the rat

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Abstract

Objectives: The therapeutic utility of hypoxia-inducible factor-1 (HIF-1) transcriptional regulatory system for ischemic hindlimb has been demonstrated. It is not yet known whether this transcriptional regulatory system can be used as a therapeutic strategy to enhance collateral vessel formation in myocardial tissues, where acute hypoxia occurs due to inadequate perfusion. We aimed to test the hypothesis that exogenous administration of HIF-1α/VP16 could enhance collateral vessel formation in a rat acute myocardial infarction model.

Methods: Sprague–Dawley rats received ligation of the proximal left anterior descending coronary artery to induce acute myocardial infarction. Immediately after the ligation, 50 μg total plasmid DNA (control, plasmid encoding human vascular endothelial growth factor (pVEGF), or pHIF-1α/VP16) was injected into the infarct area at three locations.

Results: Reverse transcription–polymerase chain reaction (RT–PCR) showed the presence of HIF-1α and VEGF mRNA in the myocardium, but not in other organs at days 3 and 7. The infarct size significantly decreased from 37±4% (control) to 24±2% in the VEGF-treated group and 23±2% in the HIF-1α/VP16 treated group (P<0.05). Capillary density also significantly increased from 550±75/mm² (control) to 850±75/mm² in the VEGF group and 850±50/mm² in the HIF-1α/VP16-treated group (P<0.01). Combined therapy with HIF-1α/VP16 and VEGF resulted in higher capillary density (1230±50/mm²) than treatment with either therapy alone. Regional myocardial blood flow was also higher in the treated groups than in the control. Plasma levels of VEGF were also significantly higher in the HIF-1α/VP16 and VEGF-treated group than in the control group.

Conclusions: The HIF-1α/VP16 hybrid transcription factor is able to reduce infarct size and enhance neovascularization in an acute ischemic myocardium. The potency of VEGF and HIF-1α/VP16 hybrid as therapeutic angiogenic factors in acute hypoxic myocardium is similar.

Keywords: Angiogenesis; Collateral circulation; Gene therapy; Infarction

1. Introduction

New blood vessel formation has been recognized as an adaptive response to cellular hypoxia [1]. In the case of local hypoxia due to inadequate perfusion (ischemia), angiogenesis is stimulated by the production of vascular endothelial growth factor (VEGF), a well-known phenomenon in coronary artery disease [2], tumor angiogenesis [3,4], and diabetic neovascularization [5]. Hypoxia also plays a significant role in regulating angiogenesis and vasoformation during mammalian embryonic development [6]. VEGF is a protein that is essential for angiogenesis [7]. Increased expression of VEGF gene in hypoxic cells is mediated in part by increased gene transcription [8–11]. Transcription of genes encoding erythropoietin, VEGF, and glycolytic enzymes is activated in hypoxic cells by a common molecular mechanism [12]. Transcriptional activation of these genes is mediated by the binding of...
hypoxia-inducible factor 1 (HIF-1) to cis-acting hypoxia-
response elements located primarily within 5′-flanking
regions of these genes [13]. These observations suggest
that activation of HIF-1 may be involved in the regulation
of vascular growth and cellular metabolism.

HIF-1, a DNA binding complex first identified as a
factor critical for the inducible activity of the erythro-
poietin 3′ enhancer [14], is a key physiological regulator of
gene expression that responds to changes in cellular
oxygen tension [15]. HIF-1 is a heterodimeric DNA
complex composed of two basic helix-loop-helix Per-
AHR-ARNT-Sim-proteins (HIF-1α and HIF-1β) [16].
HIF-1α protein levels, which determine the level of HIF-1
DNA-binding and transcriptional activity, increases ex-
ponentially as cellular oxygen concentration is reduced
[17]. Under hypoxic conditions, both HIF-1α protein level
and the activity of the HIF-1α transactivation domains
increase [17,18]. Pugh et al. demonstrated that sequences
from HIF-1α but not HIF-1β convey hypoxia-inducible
activity when fused to the DNA binding domain of heterologous transcription factors [19]. The HIF-1 gene
has been shown to be involved in tumor angiogenesis and
growth [20–22]. The therapeutic utility of this transcription-
regulatory system for targeting gene expression at hypoxic
tumor cells or ischemic hindlimb has been demonstr-
ated [22,23]. However, it is not known whether this
transcriptional regulatory system can be used as a ther-
apeutic strategy to enhance collateral vessel formation in
myocardial tissues where acute hypoxia occurs due to
inadequate perfusion.

The HIF-1α/VP16 hybrid is a constitutively active and
more potent form of HIF-1α by constructing a hybrid
transcription factor consisting of the DNA-binding and
dimerization domains from HIF-1α and the transactivation
domain from herpes simplex virus VP16 protein [24]. The
HIF-1α/VP16 hybrid up-regulates exogenous VEGF ex-
pression in vitro and enhances angiogenesis in rabbit
hindlimb ischemia [23].

Accordingly, we ought to test the hypothesis that
exogenous administration of HIF-1α/VP16 hybrid could
enhance collateral vessel formation in a rat acute myocar-
dial infarction model and to compare the potency of
HIF-1α/VP16 hybrid to that of VEGF as a therapeutic
angiogenic factor.

2. Methods

2.1. Production of acute myocardial infarction

Male Sprague–Dawley rats weighing 250–350 g were
intraperitoneally anesthetized with sodium pentobarbital
(45 mg/kg). The rats were intubated and ventilated with a
volume-cycled small-animal ventilator. An anterior
thoracotomy was performed to open the pericardium. The
heart was then rapidly exteriorized, and a 6-0 silk suture
was tightened around the proximal left anterior descending
coronary artery (before the first branch of diagonal artery).
Positive end-expiratory pressure was applied to fully
inflated the lungs. The muscle layer and skin were closed
separately after plasmid injection, and the animals were
allowed to recover. The experimental protocol was ap-
proved by the Shin Kong WHS Memorial Hospital com-
mittee on animal experiments.

2.2. Plasmids

The HIF-1α/VP16 plasmid was generated by Genzyme
Corporation (Framingham, MA, USA). cDNA fragments
of HIF-1α at amino acid (aa) 390 as well as VP16 C-
terminal aa 413–490 were assembled into a simple eukary-
otic expression plasmid that uses a CMV promoter to drive
HIF-1α expression. Downstream from the HIF-1α cDNA
is a BGH polyadenylation sequence. These fragments
occur in the pUC19 vector, which includes an SV40-neo
gene for neomycin resistance. The plasmid pCMVβ encod-
ing β-galactosidase under control of CMV promoter/en-
hancer was used for the control transfection experiments.
Plasmids were purified with QIAGEN kits. Ethanol
precipitation was used to sterilize all plasmids in preparation
for myocardial injection, after which, the DNA pellets
were reconstituted with sterile PBS containing 5% sucrose,
and stored at −20°C. Prior to injection, DNA concen-
trations were determined by a spectrophotometer. The
plasmid constructs pHGF165 and pCMVβ have been
previously described [25].

2.3. Intramyocardial gene transfer

After ligation of the left anterior descending coronary
artery, 50 μg of total plasmid DNA in 0.1 ml of normal
saline were injected intramuscularly at the left anterior free
wall by using an insulin syringe with a 30-gauge needle.
After the left ventricle was accessed, the needle was
advanced along the left ventricular free wall and plasmid
DNA was injected over a period of 5 to 10 s at three
separate sites. The injected sites were chosen at least 5 mm
away from the left ventricular apex. All animals received
three intramyocardial injections of plasmid, with the
control plasmid being pCMVβ. In the treated animals,
PHIF-1α/VP16 or pHGF165 was injected. After injec-
tion, the chest was closed and the animals were allowed
to recover.

2.4. β-Galactosidase gene expression

To localize the areas of injection and reporter gene
expressions relative to the infarcts, β-galactosidase activity
was assessed by incubating muscles in 5-bromo-chloro-3-
indolyl-β-d-galactosidase chromogen (X-Gal, Sigma, St.
Louis, MO, USA) overnight at 37°C after intramyocardial
injection of pCMVβ in four additional rats. After staining,
the muscles were rinsed in saline, post-fixed in 1% paraformaldehyde, and were then paraffin embedded, sectioned, and counterstained with hematoxylin and eosin. Five sections from each sample were randomly selected, and the numbers of positive and total myocytes in five high-power fields among an area including pericardium were counted manually for each specimen.

2.5. Human VEGF and HIF-1 gene expression in ischemic myocardial muscles

Gene expression was evaluated by detecting mRNA levels using RT–PCR with rats of myocardial infarction that were put to death at days 1, 3, 7, 14 and 28 after the transfection with pCAGGS-VEGF165 and HIF-1α/VP16 hybrid (n=2 at each time point) or control plasmid (n=2 at 7 days after transfection). In the eight rats killed at 3 and 7 days after transfection, remote tissues (lung, liver, spleen, and aorta) were also retrieved for analysis of human VEGF and HIF-1α mRNA. To ensure specificity and avoid amplification of endogenous rat VEGF and HIF-1α, each primer was selected from a region that was not conserved among different species. Sequences of primers used for VEGF were 5'-GAGGCGAATTCATCAGAAGT-3' (sense) and 5'-TGAGAGATCTGGTTCCCGAAAC-3' (antisense). Sequences of primers used for HIF-1α were 5'-AGAAAAAGATATGGATTTCCCGAAAC-3' (sense) and 5'-GAGAAAAAGCTTCGCTGTGTG-3' (antisense). RT–PCR was performed according to the manufacturer’s protocol (Access RT–PCR System, Promega, Madison, WI, USA). The size of the PCR product for VEGF and HIF-1α was 531 and 478 bp, respectively. RT–PCR products were analyzed by 2% agarose gel electrophoresis. To detect the endogenous gene response to the ischemic change, real time PCR using a Lightcycler (Roche Diagnostics, Mannheim, Germany) was performed with rat specific primers for VEGF and HIF-1α. Sequences of primers used for rat VEGF were 5'-CACCCAC-GACAGAAGG-3' (sense) and 5'-TCACAG-TGAACGCTCC-3' (antisense). Sequences of primers used for rat HIF-1α were 5'-AGTCGGACAGCCTCAC-3' (sense) and 5'-TGCTGGCTTTGTATGGGA-3' (antisense). The initial denaturation phase was 10 min at 95°C followed by an amplification phase as described below: denaturation at 95°C for 10 s; annealing at 55°C for 5 s; elongation at 72°C for 15 s and for 30 cycles. Amplification, fluorescence detection, and post-processing calculations were also performed using the Lightcycler apparatus.

2.6. Infarct size determination

Two weeks after myocardial infarction, rats were deeply anesthetized with pentobarbital and put to death by rapid excision of the heart. The atria were trimmed from the ventricles, and the right ventricle and left ventricle plus septum were separated and weighed. The tissues were then immersed and fixed in 10% buffered formalin. Each heart was sliced in cross section at four levels spanning from the apex to the base and prepared for routine histology. These sections from each level were stained with 1% triphenyltetrazolium chloride (TTC) for 20 min. The histological sections of all four slices were projected on a digitalization screen. A planimeter was used to obtain the length of the entire endocardial circumference and that segment of the endocardial circumference made up by the infarcted portion from each of the four slices of the left ventricle. The infarct size, expressed as a percentage of the left ventricle including the septum, was calculated by dividing the circumference of the infarct by the total circumference of the left ventricle including the septum. The person who measured the infarct size was unaware of the treatment group.

2.7. Capillary density by immunohistochemistry

At the day of sacrifice (days 14 and 28 after transfection, respectively), the left ventricle was harvested, fixed in methanol, and sliced into 5 μm paraffin sections. To block endogenous peroxide activity and nonspecific binding, sections were incubated with 3% hydrogen peroxide followed by 10% normal horse serum. Specimens were incubated with a monoclonal anti-mouse CD31 antibody at 4°C overnight. Bound primary antibodies were detected with the avidin–biotin–immunoperoxidase method (Sigtanics, Mannheim, Germany) was performed with rat specific primers for VEGF and HIF-1α. Sequences of primers used for rat VEGF were 5'-CAGCAGAAGG-3' (sense) and 5'-TCACAG-TGAACGCTCC-3' (antisense). Sequences of primers used for rat HIF-1α were 5'-AGTCGGACAGCCTCAC-3' (sense) and 5'-TGCTGGCTTTGTATGGGA-3' (antisense). The initial denaturation phase was 10 min at 95°C followed by an amplification phase as described below: denaturation at 95°C for 10 s; annealing at 55°C for 5 s; elongation at 72°C for 15 s and for 30 cycles. Amplification, fluorescence detection, and post-processing calculations were also performed using the Lightcycler apparatus.

2.8. Myocardial perfusion detection

At day 28 after transfection, animals were reanesthetized and their chests were opened. Radioactive microspheres (PerkinElmer Life Sciences, Inc., Boston, MA, USA), approximately 700 000 in number, labeled with 141Cr, were injected into the left ventricular cavity. The microspheres were allowed to circulate for 1 min. Then, the animals were euthanized, and the hearts were excised. Tissue specimens from the left ventricle including the septum and right ventricular free wall were excised for measurement of radioactivity. The activities in the left ventricle were expressed per weight of heart tissue as a ratio relative to the activity in the right ventricular free wall.
2.9. Measurement of plasma VEGF

For the first six rats in each group, blood samples were drawn from the left carotid artery at day 3 and from the right femoral artery at day 7 using a 23-gauge needle after coronary ligation. After sampling 0.5 ml of blood, the left carotid and right femoral arteries were ligated. The blood sample was stored at 4 °C for 30 min and then centrifuged at 3000 rev./min for 15 min. Plasma was frozen at -80 °C until assay of VEGF by a mouse VEGF ELISA kit, purchased for R&D Systems. The lower limit of detection of plasma VEGF was 5 pg/ml. The assay was performed in duplicate for each sample.

2.10. Statistical analysis

All results were expressed as mean±S.E.M. Statistical significance was evaluated by analysis of variance followed by Scheffe’s procedure. A value of $P<0.05$ was considered to denote statistical significance.

3. Results

3.1. Evidence of transfection

To demonstrate the activity of reporter genes, X-Gal staining was performed 4 days after intramyocardial injection. In the myocardium transfected with pCMVβ, successful transfection was evidenced by dark-blue stains that were observed at the area near the infarct size, but not in the infarct area (Fig. 1). This evidence showed that the plasmids were delivered into the peri-infarct zone. The efficiency of gene transfer using β-galactosidase detection was 3.6±0.5%.

3.2. Human HIF-1α/VP16 and VEGF gene expression in myocardium

To confirm HIF-1α/VP16 and VEGF gene expression in the transfected rat myocardium, we analyzed the gene expression of transfected myocardium by detecting mRNA

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**Fig. 1.** Expression of β-galactosidase in the ischemic myocardium. Positive expression is identified as dark-blue staining of myocytes (arrow).

**Fig. 2.** Expression of human HIF-1α and VEGF mRNA by RT-PCR in ischemic myocardium. (A) Human HIF-1α and VEGF mRNA expression at different periods of time. M, Promega 100-bp DNA ladder; H+, V+, positive control for HIF-1α and VEGF, respectively; H1, H2, H4, H8, and H28, ischemic myocardium transfected with pHIF-1α/VP16 at days 3, 7, 14, and 28; V1, V3, V7, V14, and V28, ischemic myocardium transfected with pVEGF165 at days 1, 3, 7, 14 and 28; Corresponding β-actin expression is indicated below. (B) No expression of human HIF-1α and VEGF mRNA at extramyocardial tissues. M, Promega 100-bp DNA ladder; +, positive control; 1, 4, 7, 10, and 13, brain, lung, liver, kidney and limb muscle, respectively, from rats transfected with pHIF-1α/VP16 at day 7; 2, 5, 8, 11, and 14, brain, lung, liver, kidney and limb muscle, respectively, from rats treated with pVEGF165, 3, 6, 9, 12, and 15, same tissue as mentioned above from rats treated with pCMVβ. Corresponding β-actin expression is indicated below.
using RT–PCR. HIF-1α and VEGF mRNA were detected in the myocardium at days 1, 3, 7 and 14 after gene transfection (Fig. 2). The mRNA of HIF-1α and VEGF could not be detected at day 28 after transfection. Neither HIF-1α nor VEGF mRNA was detected in distal tissues at days 3 and 7. Rat myocardium injected with pCMVβ plasmid was consistently negative for HIF-1α and VEGF mRNA. To detect the endogenous gene response to the ischemic change, real time PCR using a Lightcycler (Roche Diagnostics, Mannheim, Germany) was performed with rat specific primers for VEGF and HIF-1α. The mRNA of endogenous VEGF at day 7 after infarction in the pHIF-1α/VP16, phVEGF_{165}, and pCMVβ-treated groups increased 2.4-, 4.2-, and 2.1-fold, respectively as compared to the normal rat without infarction. The mRNA of endogenous HIF-1α at 7 days after infarction in the pHIF-1α/VP16, phVEGF_{165}, and pCMVβ-treated groups increased 4.3-, 3.8-, and 2.8-fold, respectively as compared to the rat without infarction.

### 3.3. Infarct size

Since the infarct size of the rats transfected with pCMVβ was similar to that of rats transfected with saline only (data not shown), we used pCMVβ-treated rats as the control. A total of 44 rats were studied for the determination of infarct size with surgical mortality rates after coronary ligation and intramyocardial injection at 31% in each group. Measurement of the infarct size of the left ventricle was performed in 31 hearts (four pHIF-1α/VP16 plus phVEGF_{165}-treated hearts, nine pHIF-1α/VP16-treated hearts, nine phVEGF_{165}-treated hearts and nine controls) at day 14 after transfection. Infarct size was significantly smaller in the pHIF-1α/VP16-treated and phVEGF_{165}-treated groups than in the control group (23±2 and 24±2% vs. 37±4%, P<0.01 and P<0.05, respectively) as shown in Fig. 3. The infarct size was similar between the HIF-1α/VP16-treated and VEGF-treated groups. The infarction ratio (20±3%) was further reduced by the combination of pHIF-1α/VP16 and phVEGF_{165}. The infarct size was also significantly smaller in the pHIF-1α/VP16 and phVEGF_{165}-treated groups than in the control at day 7 after transfection (data not shown).

### 3.4. Capillary density

Given that CD31 antibody specifically identifies capillaries, we used CD31 antibody to detect capillaries at 14 and 28 days after transfection. Capillary densities observed in the myocardium of the pHIF-1α/VP16-treated group (850±50/mm²) and the phVEGF_{165}-treated group (850±75/mm²) were significantly higher (P<0.01) than that of the control (550±75/mm²) at day 28 after transfection, as shown in Fig. 4. The capillary density in the group of combined pHIF-1α/VP16 and phVEGF_{165} was significantly higher than that of either pHIF-1α/VP16-treated or phVEGF_{165}-treated group. The capillary density was similar between the HIF-1α/VP16-treated and VEGF-treated groups. The capillary density of the control group (pCMVβ) was significantly higher than that of the rat without infarction (data not shown). The capillary densities at day 14 after transfection were also significantly higher (P<0.05) in the pHIF-11α/VP16-treated group (650±50/ mm²) and the phVEGF_{165}-treated group (625±50/mm²) than in the control (400±75/mm²). Grossly and microscopically, no angiomina formation was found in any treated-animals or controls. The increased capillary density was mainly limited to the area around the infarct area (border zone).

### 3.5. Measurement of regional blood flow

Radioactive microspheres were used to measure relative blood flow in four hearts in each group. The right ventricular free wall, which was noninfarcted in this model, served as the reference region. The weight of the left ventricle and the right ventricular free wall in each heart was about 1.0 and 0.2 g, respectively. In the rat without infarction, the radioactivity of the left ventricle was 2-fold that of the right ventricular free wall. As shown in Fig. 5, the regional blood flow ratio was significantly higher in the treated rats than in the control group. There was no significant difference in relative blood flow among the three treated groups.

### 3.6. HIF-1α/VP16 hybrid increases plasma VEGF level

As shown in Fig. 6A and B, the plasma VEGF level in
Fig. 4. Effect of intramyocardial pHIF-1α/VP16 and pHVEGF$_{165}$ on capillary density. The number of rats was also nine in each group. Photomicrography shows representative immunohistochemical CD31 staining of ischemic myocardium harvested at day 28. Dots (arrow) indicate capillaries.

the HIF-1α/VP16-treated group and the VEGF-treated group was significantly higher than that of the control group at days 3 and 7 after coronary ligation. There was no statistical difference in VEGF levels between the HIF-1α/VP16-treated group and the VEGF-treated group.

4. Discussion

It has been reported that a deletion mutant of HIF-1α truncated at aa 390 exhibits severely reduced transactivation activity but retains a high level of DNA binding that is equivalent in hypoxic and non-hypoxic cells [26]. This result suggests that both the activation domain and the protein region responsible for conferring destabilization with normoxia are located between aa 390 and aa 826. By deleting this region of HIF-1α and replacing it with transactivation domain of herpes simplex virus VP16, the HIF-1α/VP16 hybrid up-regulates exogenous VEGF expression in vitro and enhances angiogenesis in rabbit hindlimb ischemia [23]. In this study, we provide the first demonstration that intramyocardial administration of HIF-
Caution should be taken when interpreting our data. Since the results of histochemical staining with TTC were not validated by direct histologic examination of the affected tissues, we could not exclude the possibility that tissue staining properties were altered by therapy without prevention of tissue necrosis. Because capillary density would likely differ importantly in infarcted and noninfarcted regions, the amount of each tissue type within samples might significantly influence study outcome. Measurements of myocardial perfusion reflect blood flow to the entire left ventricle without distinction between flow to infarct and flow to surviving myocardium. The larger, better-perfused surviving portion of the myocardium is likely to dominate flow results. The observed flow increases in hearts treated with HIF-1α/VP16 or pHVEGF165 might be due to a larger portion of surviving myocardium—or to myocardial hypertrophy, or to a rise in myocardial oxygen demand—rather than to a rise in capillary density.

Expressions of human HIF-1α/VP16 and VEGF transgenes were detected by RT–PCR in the myocardium. No gene expression was detected in remote tissues, including lung, liver, kidney and brain. Thus, intramyocardial administrations of pHVEGF165 and HIF-1α/VP16 result in gene expression limited to the target site. Schwarz et al. demonstrated that angioma formation in the infracted tissue was found after intramyocardial injection of 500 μg of pHVEGF165 in a rat model of myocardial infarction [27]. We did not find any angioma formation in HIF-1α/VP16 and VEGF-treated rats in this study: angioma formation may be dose-related. In this study, 50 μg of plasmid was used. A recent study also demonstrated that high levels of VEGF expression caused angioma formation in muscle tissue, but low VEGF levels did not cause angioma formation [28]. Thus, intramyocardial injection of HIF-1α/VP16 and pHVEGF165 may be feasible in patients with acute myocardial infarction or with recent infarcts if a smaller dosage is administered.

Administration of HIF-1α/VP16 hybrid via gene therapy may prove to be an effective treatment for
ischemia associated with vascular disease. In this application, HIF-1α/VP16 may up-regulate a variety of genes, including VEGF. Clinical benefits may be achieved, not only as a result of stimulation of angiogenesis, but also through additional HIF-1-mediated local adaptations to low oxygen tension such as vasodilatation, protection from oxidant stress, and a transition to anaerobic metabolism.

This study demonstrated that intramyocardial injection of plasmid DNA encoding HIF-1α/VP16 was sufficient to enhance revascularization in a rat model of acute myocardial infarction, although no attempt was made in this study to determine the corresponding extent of functional improvement. This strategy provides an alternative method for therapeutic angiogenesis by exploiting a transcriptional regulatory system. These findings may have important practical implications for the treatment of patients with severe myocardial ischemia.

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