Angiogenesis Induced by *h*VEGF$_{165}$ Gene Controlled by Hypoxic Response Elements in Rabbit Ischemia Myocardium

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Hypoxic response element (HRE) offers satisfactory control over expression of *h*VEGF$_{165}$ in cell levels. However, the characteristics of regenerated blood vessels induced by long-term expression of transferred *h*VEGF$_{165}$ under control of HRE *in vivo* remain unknown. This study aims to investigate the effect of HRE on control of long-term expression of rAAV-delivered *h*VEGF$_{165}$ gene to ischemic myocardium and evaluate characteristics of angiogenesis induced by *h*VEGF$_{165}$ *in vivo*. Rabbit ischemic heart models were established surgically, rAAV-9HRE-*h*VEGF$_{165}$ was transfected to ischemia hearts subjected to 12 week ischemia. Molecular biological and immunohistochemistry were employed to determine expressions of HIF-1α and *h*VEGF$_{165}$. Microvessel densities of CD31$^+$ and α-SMA$^+$ regenerated vessels were also evaluated. Expressions of both *h*VEGF$_{165}$ mRNA and protein were upregulated following overexpression of endogenous HIF-1α early after ischemia, peaked at 4–6 weeks post-MI, declined, and approached pre-ischemia level at the end of 12 weeks of ischemia ($P < 0.01$). The significantly upregulated CD31 in *h*VEGF$_{165}$-treated hearts presented from 8 to 12 weeks of ischemia compared with the control ($P < 0.01$). However, α-SMA expression was rapidly downregulated after ischemia and remained lower than the control level by the end of 12 weeks post-MI ($P < 0.01$). Overexpression of *h*VEGF$_{165}$ controlled by HIF-1α-HRE system shows a stably regional angiogenic efficacy *in vivo*. But, VEGF, as an early angiogenic cytokine, is inadequate for mediating histologically mature vessels in ischemia myocardium. Exp Biol Med 234:1417–1424, 2009

Key words: vascular endothelial growth factor (VEGF); angiogenesis; hypoxic response element (HRE); gene control; myocardial ischemia

Introduction

Increasing numbers of investigations have been performed on angiogenic factor therapy for ischemic heart disease (1, 2). Animal studies and clinical data indicate that factors such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) are capable of inducing angiogenesis in ischemic myocardium and improving heart performance (3–8). Gene therapy strategies that use recombinant Adeno-associated viruses (rAAV) vector in order to achieve sustained local delivery of angiogenic proteins have offered a promising approach for ischemic heart therapy (9, 10).

However, uncontrolled long-term expression of VEGF delivered by rAAV vector *in vivo* may well result in certain unwanted side effects, such as hemangioma formation, retinopathy, arthritis, occult tumor growth, and atherosclerotic plaque progression (11). Thus, development of an effective gene control system that prevents these side effects of long-term VEGF expression in the setting of ischemic heart disease is necessary (12).

In our previous studies, we reported that hypoxic response element (HRE), an oxygen-mediated gene switch, offered satisfactory control over the expression of a human VEGF$_{165}$ (*h*VEGF$_{165}$) gene in both cell levels and short-term transfer in an animal model (13–15). However, the histological characteristics of regenerated blood vessels induced by long-term expression of transferred *h*VEGF$_{165}$ under control of HRE *in vivo* remain unknown and should be further elucidated.

In the present study, a concatemer of nine copies of the

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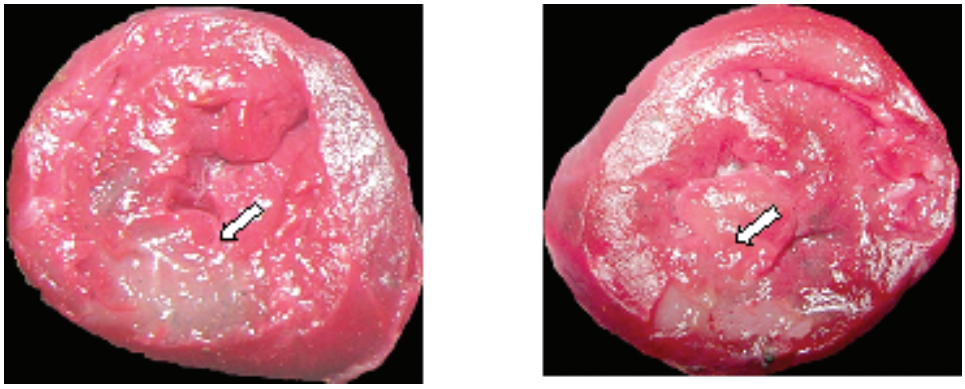


Figure 1. Morphology of cross-section of left ventricle after LAD ligation. The arrows indicate the irregular myocardial infarction focus located at anterior wall of left ventricle with the obviously local tissue edema. A color version of this figure is available in the online journal.

consensus sequence of HRE isolated from the erythropoietin enhancer was employed to mediate hypoxia induction (16). We used molecular biology, immunohistochemistry, and morphological analysis to investigate the effect of HRE on regulating the long-term expression of rAAV-delivered hVEGF$_{165}$ gene to the rabbit ischemic myocardium, focusing on evaluating the characteristics of angiogenesis induced by hVEGF$_{165}$ *in vivo*.

Materials and Methods

Recombinant AAV Vector. Recombinant AAV vectors were prepared with a three-plasmid co-transfection system by the method described previously (14). The rAAV vector with two helper plasmids was co-transfected to HEK293T cells using the calcium phosphate precipitation method. One helper plasmid contained the adenoviral VA, E2A, and E4 regions, which mediate AAV vector replication, while the other contained AAV rep and cap genes. The rAAV vector was purified to approximately 80%–90% by NaCl-chloroform/chloroform-PEG8000, as described by Li *et al*. (17).

Animals and the Ischemic Heart Model. New Zealand rabbits (6–8 months old, weighing 2–2.5kg, 2.3 ± 0.15kg) were obtained from the Experimental Animal Centre of Xuzhou Medical College. All animals received humane care in compliance with the Guideline for Care and Use of Laboratory Animals, published by Jiangsu Province, P.R. China.

After venous anesthesia induction with Ketamine (10mg/kg), the rabbits were anesthetized with 2% barbital sodium intravenously. With tracheal intubation, respiration support was initiated by a volume-controlled ventilator (Zhongyuan Med Inc., China). A midsternal thoracotomy was performed to expose the anterior surface of the heart. The proximal left anterior descending coronary artery (LAD) was identified, and a 6.0 surgical suture (Ethicon) was placed around the artery and ligated permanently. Myocardial ischemia was confirmed by local myocardial cyanosis, and a deep S wave on the electrocardiogram (ECG) appeared immediately after LAD interruption.

According to the experimental procedures, the animal models were randomly divided into groups A, B, C, and D. Group A: rabbits treated with rAAV-H9-hVEGF$_{165}$ and receiving rAAV-H9-hVEGF$_{165}$ transfection ($n = 42$); Group B: rabbits treated with phosphate-buffered saline (PBS) and receiving PBS instead of rAAV-H9-hVEGF$_{165}$ as ischemia control ($n = 42$); Group C: rabbits treated with rAAV-H9-LacZ and receiving rAAV-H9-LacZ transfection as the vector control ($n = 42$); Group D: the sham-operated group, a surgical control group in which the animals were subjected to the same surgical procedures, except the LAD was not ligated ($n = 21$).

Intramyocardial Gene Delivery. A 1×12^{12} of rAAV-H9-hVEGF$_{165}$ vector in a final volume of 40μL of PBS (pH 7.4) was delivered intramyocardially with a 0.5-ml syringe and 25-gauge needle into four sites around the marginal zone of the ischemia myocardium. Control animals received an equivalent volume of either PBS or rAAV vector expressing the LacZ reporter gene. After gene delivery, the exposed heart was monitored for 5 minutes for resumption of sinus rhythm. The sternum incision was closed, and the animals were allowed to recover.

Based on the observation time intervals we designed, each group was re-divided into seven subgroups (6 in each group, with the exception of the sham-operated group, which had 3). The time intervals were 2 weeks, and the total observation period was 12 weeks. At each observation time, animal hearts from the related subgroup were harvested under inhalation anesthesia and fixed with liquid nitrogen and 10% neutral formaldehyde for further analysis. The hepatic tissues from groups A and B were also obtained for molecular biological analysis.

Real-Time Quantitative Polymerase Chain Reaction (RT-Q-PCR). The ischemic myocardium was flash-frozen in liquid nitrogen for subsequent extraction and purification of mRNA, followed by RT-PCR Taqman assay to evaluate the mRNA expression of HIF-1α and hVEGF$_{165}$. Proprietary probes were purchased from ABI and sequences from Shengon Bio Inc. (China). Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA), as

described by the manufacturer. Proprietary sequences were purchased from Shengon Bio Inc. (China).

Total RNA was digested with DNase I (Invitrogen, Carlsbad, CA) at 37°C for 15 minutes RNA concentrations were determined by measuring the absorbance at a wavelength of 260 and 280 nm. 2-μg of total RNA was reverse transcripted with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) following the instructions provided.

Samples were subjected to real-time PCR in triplicate on an ABI 7300 sequence detection system (Applied Biosystems Inc., Foster City, CA), with β-actin as internal control.

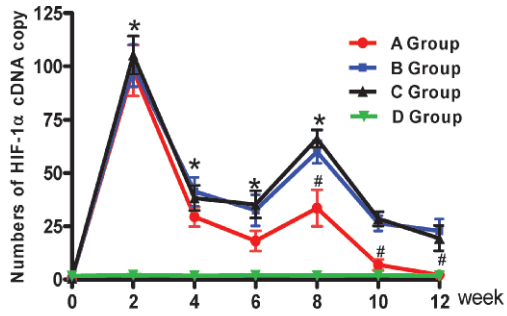
The primer sequences of HIF-1α were: 5′-CATGCCCCAGATTCAAGATCA-3′ for the forward primer and 5′-TGGTAGGCTCAGGTGAACTCTGT-3′ for the reverse primer. The primer sequences of *h*VEGF$_{165}$ were 5′-CTACCTCCACCATGCCAAGTG-3′ for the forward primer and 5′-GCAGTAGCTGCGCTGATAGACA-3′ for the reverse primer.

Western Blot Assay. One hundred milligrams of ischemic myocardium and hepatic tissues were prepared, and 1ml of cooled protein extract solution was added after homogenization (8000*g*, 10s, ×15). The content of protein was quantified by Lowry's method.

Expressions of *h*VEGF$_{165}$ and HIF-1 protein were evaluated by immunoblotting. Three myocardial samples from each subgroup of animals were determinated. Briefly, a 100-mg protein sample was separated by electrophoresis though 10% SDS-PAGE electrophoresis and then transferred to a nitrocellulose membrane (pore size 0.45μm, 3.0 mA/cm^2 for 40min). After that, the membrane was blocked for 3 hours at room temperature, and the mouse monoclonal antibodies against human *h*VEGF$_{165}$ (1: 250, Sigma Inc., St. Louis, MO) and against rabbit HIF-1α (1:250, Sigma Inc.) were added at 4°C overnight. After washing with the buffer, the secondary sheep anti-rat IgG-AP antibody conjugated with alkaline phosphatase (1:100, Zhongshan Bio Inc., China) was added for 2 hours at room temperature. The coloration of NBT/BCIP was performed, and imaged bands were scanned and semi-quantitatively analyzed (UVP Inc., California).

Immunohistochemical Stain. The tissue slices of myocardium from two sides of the ischemia marginal zone were processed. The immuno-histochemical stain was carried out based on the manufacturer's instructions (Zhongshan Bio Inc., China). Briefly, the sections were incubated with a rat monoclonal antibody against human VEGF$_{165}$ and the rat monoclonal antibodies were incubated against rabbit CD31 (1:50, Zhongshan Bio Inc., China) and rabbit α-SMA (1:50, Zhongshan Bio Inc., China) at 4°C overnight. After a simple washing with PBS, the sections were incubated with the secondary goat anti-rat IgG antibody (1:200, Sigma Inc., St. Louis, MO) at 4°C overnight and then incubated with peroxidase for 1 hour at 37°C. Afterwards, DAB coloration was carried out.

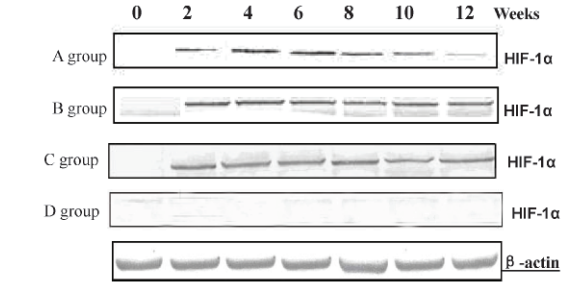
Microvessel Density Measurement. Microvessel density (MVD) of myocardial tissue sections in the peri-infarct area was evaluated by the method described previously (18, 19). At low power field (×40), the sections were scanned and five areas with the highest positive CD31 or α-SMA vascular density (hotspots) were selected. Microvessel counts of these areas were performed at high power field (×200) with a computer image analyzer

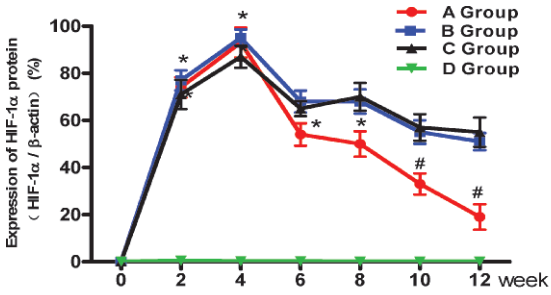


a. Expression of HIF-1mRNA in ischemia myocardium

* *P*<0.01 *vs.* pre-ischemia level ;

# *P*<0.05 *vs.* relevant point in Groups B and C.





b. Expression of HIF-1α protein in ischemia myocardium

* *P*<0.001 *vs.* pre-ischemia level ;

# *P*<0.05 *vs.* relevant point in Groups B and C

Figure 2. Expressions of HIF-1αmRNA and protein in rabbit myocardium. (a) Expressions of HIF-1α mRNA in groups A, B, and C, but not group D, were upregulated and peaked at 2 weeks post-MI. At 4–10 weeks post-MI, expression of HIF-1α mRNA in group A was significantly downregulated compared with groups B and C ($P < 0.01$) and almost approached pre-ischemia level by the end of myocardial ischemia ($P < 0.01$). (b) Expressions of HIF-1α protein in groups A, B, and C were upregulated and peaked at 4 weeks post-MI. At 12 weeks post-MI, expression of HIF-1α protein in group A was significantly lower than in other groups ($P < 0.05$). A color version of this figure is available in the online journal.

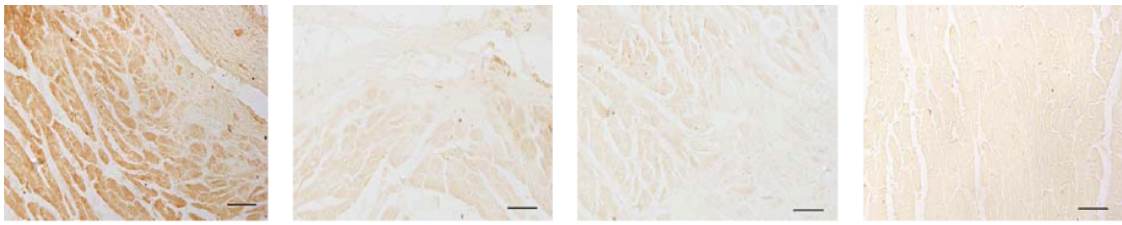


Figure 3. Immunohistochemical stain for *h*VEGF$_{165}$ protein expression in rabbit myocardium. After 4 weeks of myocardial ischemia, positively stained cells were extensively distributed in rAAV-H9-*h*VEGF$_{165}$–transfected group (A4) and no positive cells were detected in groups PBS (B4), rAAV-9HRE- LacZ (C4), and sham-operated control (D4) at the same time (bar = 50 μm). A color version of this figure is available in the online journal.

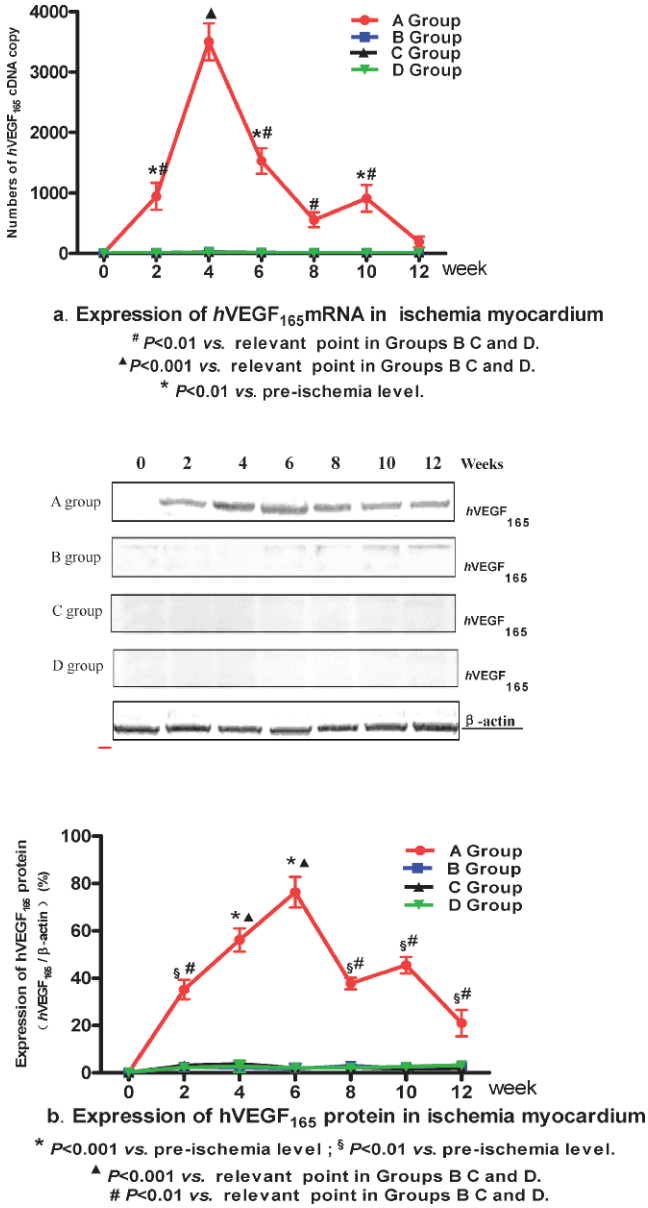


Figure 4. Expressions of *h*VEGF$_{165}$ mRNA and protein in rabbit myocardium. (a) In the rAAV-H9-*h*VEGF$_{165}$–transfected group, expression of *h*VEGF$_{165}$ mRNA was upregulated and peaked at 4 weeks post-MI, being significantly higher than any other groups ($P <$ 0.001); by the end of myocardial ischemia, *h*VEGF$_{165}$ mRNA expression had already reached its pre-ischemia level ($P > 0.05$). (b) Upregulated expression of *h*VEGF$_{165}$ protein in group A peaked (HPIAS2000 color imaging analyzer system, Tongji Qianping Imaging Co., China). An automated microvessel count per field was computed in each hot spot. The mean microvessel count of the five selected areas was taken as the MVD, which was expressed as the absolute number of microvessels per 0.74 mm^2 (×200 magnification). All measurements were performed in a blinded manner.

Statistical Analysis. All data were expressed as mean ± standard deviation (SD) and analyzed by analysis of variance (ANOVA) and Student-Newman-Keuls test using SPSS v11.0. *P* values less than 0.05 were considered statistically significant.

Results

Morphology of Ischemia Heart. Cross-sections of the ischemic myocardium from the left ventricle presented a typical pathological alteration of focal myocardial ischemia located at the anterior wall of the left ventricle, with obvious local tissue edema (Fig. 1).

Determination of HIF-1α Expression in Myocardium. Q-RT-PCR determination indicated that upregulated expressions of HIF-1α mRNA in groups A, B, and C, but not group D, were evident shortly after myocardial ischemia. In group A, HIF-1α mRNA expression peaked at 2 weeks post-MI, and then downregulated. At 12 weeks post-MI, HIF-1α mRNA expression almost approached a pre-ischemia level that was significantly lower than in groups B and C ($P < 0.01$) (Fig. 2a).

Western blot determination showed that HIF-1α protein expressions in groups A, B, and C were upregulated after initiation of ischemia. In group A, HIF-1α protein expression peaked at 4 weeks post-MI and then downregulated gradually. At 10–12 weeks post-MI, expression of HIF-1α protein in group A was significantly lower than in groups B and C ($P < 0.05$) (Fig. 2b).

Determination of *h*VEGF$_{165}$ Expression in Myocardium. Immunohistochemistry determination indicated that numerous positively *h*VEGF$_{165}$-stained cells

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at 6 weeks post-MI compared with other groups ($P < 0.01$, $P <$ 0.001), and then remained downexpressed until the end of ischemia. A color version of this figure is available in the online journal.

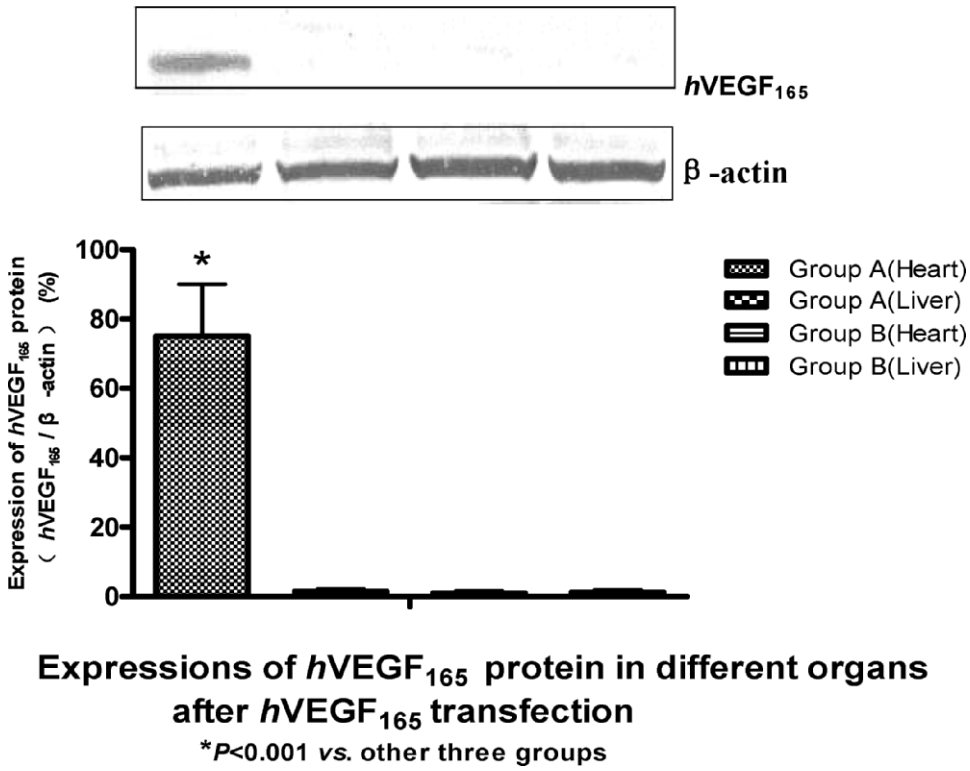


Figure 5. Expression of *h*VEGF$_{165}$ protein in different organs. Although overexpression of *h*VEGF$_{165}$ protein appeared in myocardium from group A, there was no *h*VEGF$_{165}$ protein expression in hepatic tissue collected from groups A and B at 6 weeks of myocardial ischemia.

appeared in the myocardium from group A at 4 weeks post-MI, but none was detected in groups B, C, and D at the same time (Fig. 3).

The results of Q-RT-PCR determination showed that expression of *h*VEGF$_{165}$ mRNA in group A was upregulated, peaked at 4 weeks post-MI, significantly higher than other groups ($P < 0.001$), and then, following sequential downexpression, *h*VEGF$_{165}$ mRNA expression reversed back to its pre-ischemia level by the end of 12 weeks of ischemia. However, in groups B, C, and D, *h*VEGF$_{165}$ mRNA expressions at each observation point did not differ statistically compared with their pre-ischemia levels ($P > 0.05$) (Fig. 4a).

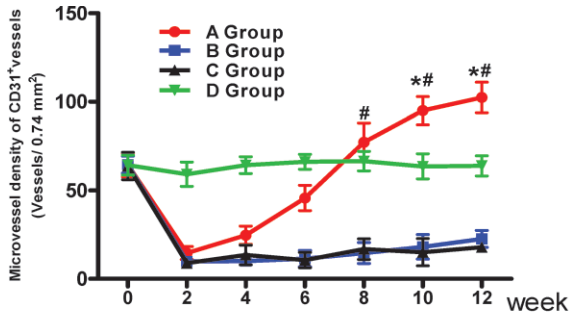
With regard to *h*VEGF$_{165}$ protein expression, in group A, a remarkable upregulation was present within 2–4 weeks, and upregulation peaked at 6 weeks post-MI; then, *h*VEGF$_{165}$ protein expression remained at a low level until the end of ischemia ($P < 0.01$, $P < 0.001$). There was no significant *h*VEGF$_{165}$ protein expression in the ischemic myocardium from groups B, C, or D at any observation point (Fig. 4b) or in the hepatic tissue collected from groups A and B at 6 weeks post-MI (Fig. 5).

Histological Analysis of Microvessel Density. Microvessel density determination indicated that the numbers of CD31$^+$ vessels in groups B and C were decreased 2 weeks post-MI and remained at much lower levels without significant enhancement until the end of ischemia, whereas in group A, the number of CD31$^+$ vessels was significantly higher than its pre-ischemia level or those in groups B, C, and D by the end of ischemia ($P < 0.01$; $P < 0.05$) (Fig. 6a,b). The result also indicated that the numbers of α-SMA$^+$ vessels in groups A, B, and C were rapidly decreased after 2 weeks of myocardial ischemia and remained at significantly lower levels until the end of ischemia, when compared with their pre-ischemia levels ($P < 0.01$) (Fig. 7a, b).

Discussion

Research on the VEGF promoter has demonstrated that a single HRE is located at nucleotide positions 947 to - 39 (5-TACGTG-3) related to the common transcription start site (20, 21). In cells and tissues, hypoxia triggers a multifaceted adaptive response that is primarily driven by hypoxia-inducible factor 1α (HIF-1α). In anoxic and hypoxic environments, HIF-1α is stabilized enough to form functional HIF-1α protein, which, in turn, binds to HREs in the enhancers of genes encoding glucose transporters and glycolytic enzymes and upregulates the gene expressions of, for example, VEGF, inducible nitric oxide synthase (iNOS), and erythropoietin (Epo). However, under a normoxia condition, the transcription activity of HIF-1α depresses quickly (22). Thus, an ideal gene control approach should be able to preserve the response of angiogenic growth factors toward hypoxia (23, 24).

Analyzing the relationship between HIF-1α and



a. Determination of CD31$^+$ microvessel density in rabbit ischemia myocardium

* P<0.05 *vs.* pre-ischemia level and group D ;

\# P<0.01 *vs.* relevant point in Groups B and C.

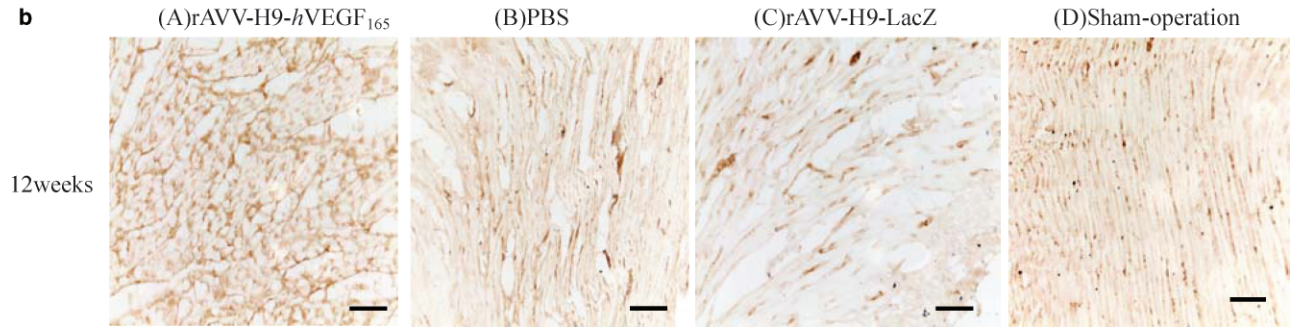


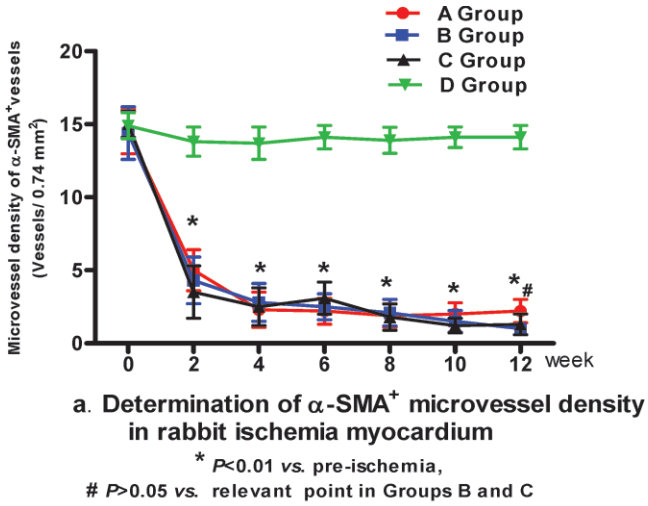
Figure 6. a. The MVD determination of CD31$^+$ vessels in rabbit myocardium. The numbers of CD31$^+$ vessels in groups B and C were decreased 2 weeks post-MI and remained at low levels without significant enhancement until the end of ischemia. The number of CD31$^+$ vessels in group A was significantly higher than its pre-ischemia level and those in other groups by the end of ischemia ($P < 0.01$, $P < 0.05$). b. Representative CD31 immunohistochemistry stained sections in rabbit ischemic myocardium (from left to right). (A) The section presented extensively distributed CD31 positively stained cells following delivery of rAAV-9HRE-hVEGF$_{165}$ at 12 weeks post-MI compared with groups B and C. (B) A few of the CD31 positively stained cells appeared following administration of PBS after 12 weeks of ischemia. (C) Only fewer CD31 positively stained cells were detected following delivery of rAAV-9HRE- LacZ at 12 weeks post-MI. (D) The section presented that uniformly arranged CD31 positively stained cells were distributed in myocardium from sham-operated control (bar = 100 μm). A color version of this figure is available in the online journal.

hVEGF$_{165}$ expressions in our study, we noted that the peaked expressions of hVEGF$_{165}$ productions occurred approximately two weeks later than those of HIF-1α. This slightly deferred expression of hVEGF$_{165}$ productions signified that the HIF-1α-HRE control system responded in a timely and sensitive manner to myocardial hypoxia. On the other hand, downexpression of hVEGF$_{165}$ by the end of ischemia indicated that the local myocardial ischemia was ameliorated by the hVEGF$_{165}$-induced angiogenesis.

Focusing on regenerated vessels, we considered that the severe ischemia or hypoxia was certainly responsible for impairing native vascular endothelium, and impairment contributed to decreased CD31$^+$ MVD shortly after myocardial ischemia. Theoretically, angiogenesis must be mediated by a valid overexpressed VEGF protein. In our study, we found evidence that enhanced CD31$^+$ MVD had already approached the pre-ischemia levels at 6 weeks post-MI and followed only the peaked expression of hVEGF$_{165}$ productions. This finding indicated that the peak time of hVEGF$_{165}$ expression represented an initiation of curative hVEGF$_{165}$-induced capillary development. Moreover, by the end of 12 weeks of ischemia, CD31$^+$ MVD in hVEGF$_{165}$-treated hearts was 1.5 times higher than the pre-ischemia level because of the delayed disappearance of hVEGF$_{165}$ protein and an accumulation of newly recruited endothelial cells. This phenomenon was helpful in our evaluation of the whole period of VEGF gene therapy in ischemic hearts. Our results indicated that effective hVEGF$_{165}$ expression during the period of time we observed led to a significant development of capillaries under control of the HRE-HIF-1 system.

Switching to α-SMA, we detected that, unlike the case of CD31, α-SMA expression after hVEGF$_{165}$ delivery, instead of being upregulated, remained significantly down-regulated during the whole period of ischemia, indicating that hVEGF$_{165}$-induced regenerated vessels were deficient in vascular basilar membrane and pericyte. The fact is that the hVEGF$_{165}$ transgene is insufficient to induce histologically mature vessels (14).

It is generally recognized that angiogenesis is a complex, multi-gene event that needs to be highly regulated and that multiple growth factors acting at different stages of angiogenesis may be required (25). Lee *et al* (26) documented that some genes with differential expressions



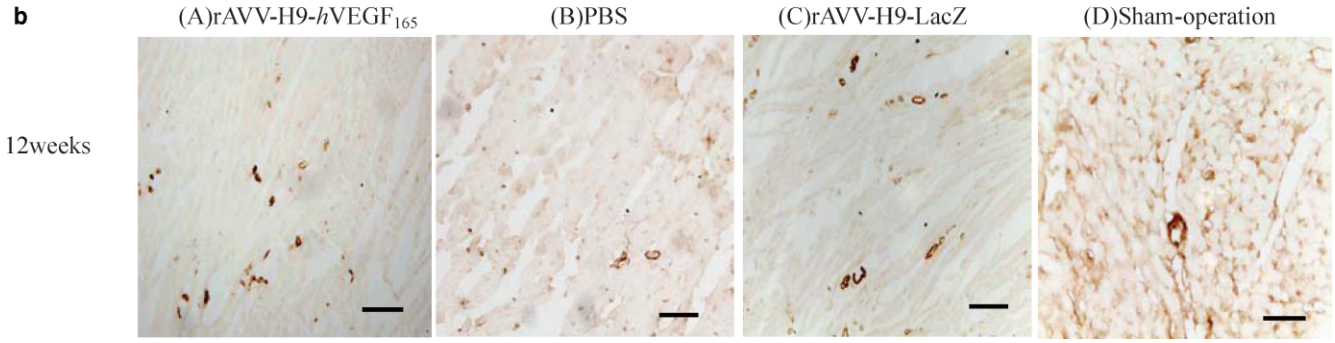


Figure 7. a. The MVD determination of α-SMA$^+$ vessels in rabbit myocardium. The numbers of α-SMA$^+$ vessels in all groups were rapidly decreased after 2 weeks of myocardial ischemia and remained significantly low until the end of ischemia, with the exception of group D ($P <$ 0.01). b. Representative α-SMA immunohistochemistry stained sections in rabbit ischemic myocardium (from left to right). (A) The rare α-SMA positively stained cells were observed in *h*VEGF$_{165}$-treated group at 12 weeks post-MI. (B) The section showed that few α-SMA positively stained cells appeared following administration of PBS at 12 weeks post-MI. (C) Resembling those in groups A and B, the section showed that following delivery of rAAV-9HRE-LacZ, only a few of the α-SMA positively stained cells existed after 12 weeks of myocardial ischemia. (D) The section presented extensively distributed α-SMA positively stained cells in myocardium from sham-operated control (bar = 100 μm). A color version of this figure is available in the online journal.

and temporal functional clustering appear to contribute to vascular collateral formation. VEGF, as an angiogenic factor acting at the early stage of angiogenesis, was originally described as a vascular permeability factor, based on its property of increasing vessel wall permeability through a Ca^{2+}-dependent pathway; this property may be responsible for producing immature vessels that are unable to create a functional collateral development (27–29). Clinically, to improve the therapeutic efficacy of long-term VEGF transgene, a modified strategy of gene transfer should be considered. Suri *et al* (30) reported that Angiopoietin-1 (Ang1), an angiogenic factor acting at the late stage of angiogenesis, was able to cooperate with VEGF by exerting a vascular endothelial barrier protective effect, blocking the permeability-increasing action of VEGF to create mature blood vessels and form a functional collateral development. In addition, Platelet-Derived Growth Factor (PDGF), an important growth factor for pericytes through PDGF-receptor, plays a significant role in blood vessel formation and the growth of blood vessels from already existing blood vessel tissue (31). Thus, we emphasize that, even though they are under the reliable control of angiogenic gene transcription system, the reasonable combination or hybrid of angiogenic factors acting separately at early or late stages of angiogenesis will overcome each other's limitations and yield an ideal synergistic effect on producing functional blood vessels for therapeutic angiogenesis.

Pathophysiologically, tissue ischemia is likely to induce endogenous VEGF expression. In fact, the angiogenesis might be mediated by both endogenous angiogenic cytokine and angiogenic transgene. Although no attempt was made in our study to evaluate endogenous VEGF expression, we found that CD31$^+$ MVD in ischemic control was slightly increased by the end of ischemia, but to a much lower level than in *h*VEGF$_{165}$-treated animals. This finding demonstrated that the effect of endogenous angiogenic cytokine involved in our study was limited and unlikely to induce significant post-ischemic angiogenesis.

As for the safety concerns of the HIF-1-HRE system, experimentally, we did not find unnecessary *h*VEGF$_{165}$ expression in any organ except the heart, and no evidence of

hemangioma formation was detected in the myocardium in which the rAAV-H9-*h*VEGF$_{165}$ gene was delivered. Nevertheless, in the present study, although *h*VEGF$_{165}$ effectively induced capillary formation under the control of the HIF-1-HRE system, the function of regenerated vessels should be further evaluated.

In conclusion, HRE is a valuable trigger for controlling long-term VEGF expression responding to tissue hypoxia. VEGF overexpression through rAAV-H9-*h*VEGF$_{165}$ gene transfer offers a stably regional angiogenic efficacy, but VEGF, as an early angiogenic growth factor, is inadequate for mediating histologically mature vessel formation in the ischemia myocardium.

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