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RESEARCH PAPER

Hypoxia-Inducible Vascular Endothelial Growth Factor Gene Therapy Using the Oxygen-Dependent Degradation Domain in Myocardial Ischemia

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ABSTRACT

Purpose A hypoxia-inducible VEGF expression system with the oxygen-dependent degradation (ODD) domain was constructed and tested to be used in gene therapy for ischemic myocardial disease.

Methods Luciferase and VEGF expression vector systems were constructed with or without the ODD domain: pEpo-SV-Luc (or pEpo-SV-VEGF) and pEpo-SV-Luc-ODD (or pEpo-SV-VEGF-ODD). *In vitro* gene expression efficiency of each vector type was evaluated in HEK 293 cells under both hypoxic and normoxic conditions. The amount of VEGF protein was estimated by ELISA. The VEGF expression vectors with or without the ODD domain were injected into ischemic rat myocardium. Fibrosis, neovascularization, and cardiomyocyte apoptosis were assessed using Masson's trichrome staining, α-smooth muscle actin (α-SMA) immunostaining, and the TUNEL assay, respectively.

Results The plasmid vectors containing ODD significantly improved the expression level of VEGF protein in hypoxic conditions. The enhancement of VEGF protein production was attributed to increased protein stability due to oxygen deficiency. In a rat model of myocardial ischemia, the pEpo-SV-VEGF-ODD group exhibited less myocardial fibrosis, higher microvessel density, and less cardiomyocyte apoptosis compared to the control groups (saline and pEpo-SV-VEGF treatments).

Conclusion An ODD-mediated VEGF expression system that facilitates VEGF-production under hypoxia may be useful in the treatment of ischemic heart disease.

KEY WORDS gene therapy · hypoxia-inducible expression · ischemic myocardium · oxygen-dependent degradation domain · vascular endothelial growth factor

INTRODUCTION

Gene therapy with angiogenic factors has recently emerged as a promising strategy for the treatment of ischemic heart disease (1,2). Among the factors that regulate angiogenesis, vascular endothelial growth factor (VEGF) plays an especially important role in the vascular endothelium, where it binds to high-affinity receptors such as tyrosine kinase FLK-1 (3). Recently it has been reported that tissue ischemia upregulates the expression of VEGF and its receptors (4), and the physiological responses to VEGF are limited to ischemic lesions with locally induced receptors. For these properties, VEGF is recognized as one of the genes potentially most effective in the therapeutic neovascularization of ischemic tissues (5,6). Studies also show, however, that unregulated continuous expression of exogenous VEGF can perturb normal and tumor tissue growth (7). Therefore, safe and effective angiogenic gene therapy with VEGF requires strategic control of expression in target tissues.

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The past decade has produced hypoxia-specific expression systems tailored for gene therapy to treat ischemic disease. For example, the erythropoietin (Epo) enhancer-simian virus 40 (SV40) and RTP801 promoters were developed to regulate transcription under hypoxic stress (8–10). Hypoxia specifically activates these transcriptional systems *in vitro,* which favors their use to promote angiogenesis in ischemic myocardium and injured spinal cord (8,11). Linkage of the hypoxia response element (HRE) to the SV40 promoter was shown to further increase gene expression in mouse ischemic myocardium (12). In addition to transcriptional control of gene expression, the regulation of mRNA stability presents another strategy for hypoxia-inducible gene expression. For instance, the 3′-untranslated region (UTR) of the Epo mRNA selectively stabilized mRNA in hypoxia by increasing mRNA translation rates (13). Expression systems with hypoxia-specific promoters encounter a drawback, however, in that their basal activity allows leaky expression under normoxia.

Hypoxia-inducible factor-1 (HIF-1) is a key transcriptional regulator of hypoxia-induced gene expression (14,15). HIF-1 acts as a heterodimer composed of an oxygen-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit (16,17). Under normoxic conditions, the HIF-1α subunit is rapidly degraded through the von Hipple-Lindau protein (pVHL)-mediated ubiquitin-proteasome pathway (18). Thus, the direct interaction between pVHL and the oxygen-dependent degradation (ODD) domain of HIF-1α plays a major role in HIF-1α degradation under normoxic conditions (19). In contrast, the HIF-1α subunit escapes the proteolysis pathway during hypoxic exposure through down-regulation of HIF prolyl hydroxylases (PHDs); this stabilizes the HIF-1 protein (20,21).

Several studies have used the ODD domain to precisely regulate the stability of proteins depending on oxygen concentration. For example, the ODD domain can stabilize diphtheria toxin in ischemic regions of solid tumors, although normal tissues readily degrade the toxin (22). Another study used the ODD domain to construct an oxygen-dependent transcriptional regulator, which was specifically stabilized in hypoxic cells and increased the transcription of target genes (23). In addition, our recent study showed that the ODD domain induces hypoxia-specific luciferase expression *in vitro* (24). These findings suggest that hypoxia-inducible gene expression systems that include the ODD domain can be used to create effective gene therapies for the ischemic myocardium.

In this study, we constructed a hypoxia-specific VEGF expression vector containing the ODD domain (pEpo-SV-VEGF-ODD) and used it to enhance VEGF expression in a hypoxia-specific manner for ischemic myocardium gene therapy. The ODD domain was expected to stabilize the VEGF protein *in vivo* and thereby increase the beneficial effects of the VEGF gene therapy. We examined the transfection efficiencies of the hypoxia-inducible VEGF expression vectors, with and without the ODD domain, in HEK 293 cells and evaluated their therapeutic potentials in a rat model of myocardial ischemia.

MATERIALS AND METHODS

Materials

pSV-Luc was purchased from Promega (pGL3-promoter, Madison, WI, USA). Polyethylenimine (branched PEI, 25 kDa) was obtained from Aldrich (Milwaukee, WI, USA). Cell culture media and materials were the products of Gibco BRL (Grand Island, NY, USA). Human embryonic kidney (HEK) 293 cells were supplied by Korea Cell Line Bank (Seoul, South Korea). All other chemicals and reagents were used as received unless otherwise mentioned.

Plasmid Construction

The human VEGF165 cDNA was used to construct the VEGF plasmids, as human VEGF is active in rat myocardium (25). The VEGF165 cDNA without the stop codon (VEGF(-)stop) was amplified by PCR using pSV-VEGF as a template. The forward and reverse primers for VEGF(-)stop were 5′-CCCAAGCTTGAAACCATGAACTTGCT-3′ and 5′-GCTCTAGACCGCCTCGGCT TGTCACA-3′, respectively. The *HindIII* and *XbaI* sites were introduced into the forward and reverse primers, respectively (the enzyme sites are underlined). The amplified VEGF(-)stop was digested with *HindIII* and *XbaI* and then purified by electrophoretic elution from a 1% agarose gel. pSV-VEGF(-)stop was constructed by insertion of the VEGF(-)stop fragment into the site of the luciferase cDNA of pSV-Luc. The ODD domain (557 to 574 GenBank accession number: AF208487) was chemically synthesized. The DNA sequence of the ODD domain was as follows: 5′-TCTAGATTAGACTTGG AGATGTTAGCTCCCTATATCCCAATGGATG ATGACTTCCAGTTATGAGGATCCGATCTAGA-3′. The *XbaI* sites were introduced at each end of the ODD domain for cloning convenience. The synthesized ODD domain was annealed and inserted into the *XbaI* site of pSV-VEGF(-)stop to produce pSV-VEGF-ODD. pEpo-SV-VEGF was constructed as previously described (8). Briefly, the Epo enhancer-SV40 promoter fragment was isolated by digestion of pEpo-SV-Luc with *NheI* and *NcoI*. The SV40 promoter of pSV-VEGF-ODD was eliminated by digestion with *NheI* and *NcoI*. The Epo enhancer-SV40 promoter fragment was inserted into the site of the SV40 promoter to

produce pEpo-SV-VEGF-ODD. The luciferase expression plasmid with the ODD domain was constructed in the same way. These plasmids were confirmed by restriction enzyme digestion and DNA sequence analysis (Fig. 1).

Cell Culture and *In Vitro* Transfection

HEK 293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS in a 5% CO_2 incubator. For *in vitro* transfection assays, the cells were seeded at a density of 1.0×10^5 cells/well in a 6-well plate. After 24 h, the cell culture medium was replaced with 2 ml of fresh serum-free medium. For *in vitro* experiments, branched PEI (25 kDa) was used as a polymeric gene carrier to form polymer/DNA complexes. PEI and DNA were complexed at an N/P (nitrogen of PEI/ phosphate of DNA) ratio of 5:1. The polymer/plasmid complexes were added to each well. The cells were then incubated for 4 h at 37°C. After 4 h for transfection, the medium was exchanged with fresh medium containing 10% FBS. The cells were further incubated at the desired concentration of oxygen (normoxia: 20% oxygen, 5% CO_2, 75% nitrogen; hypoxia: 1% oxygen, 5% CO_2, 95% nitrogen) for 20 h. Each transfection experiment was repeated at least three times to ensure its reproducibility.

Luciferase Assay

Luciferase gene expression was determined at 24 h after transfection using a commercial luciferase assay kit (Promega). The transfected cells were washed extensively with PBS and lysed by adding 200 µl of reporter lysis buffer. Luciferase activity was measured in terms of relative light units (RLU) using a 96-well plate luminometer (Berthold Detection System GmbH, Pforzheim, Germany). The luciferase activity was monitored and integrated over a period of 20 s. The relative values of luciferase activity were reported in terms of RLU/mg total protein. The protein concentration of the extract was determined using a Bradford protein assay kit (Intron Biotechnology, Seoul, Korea). All of the data are presented as the mean ± standard deviation (SD) of three independent measurements.

Enzyme-Linked Immunosorbent Assay (ELISA)

VEGF protein expression was measured *in vitro* and *in vivo* using a commercially available ELISA kit (Biosource Human Vascular Endothelial Growth Factor ELISA kit, Biosource International Inc., Camarillo, CA, USA). For *in vitro* assay, the conditioned cell culture medium was harvested and analyzed for VEGF 24 h after transfection. The gene transfection efficiencies were normalized by total protein concentration determined by Bradford protein assay kit. For *in vivo* assay, rats were sacrificed at 5 days post-injection. The myocardium was isolated and homogenized in reporter lysis buffer. The VEGF protein was quantified using the ELISA kit according to the manufacturer's instructions. All of the data are presented as the mean ± standard deviation (SD) of three independent measurements.

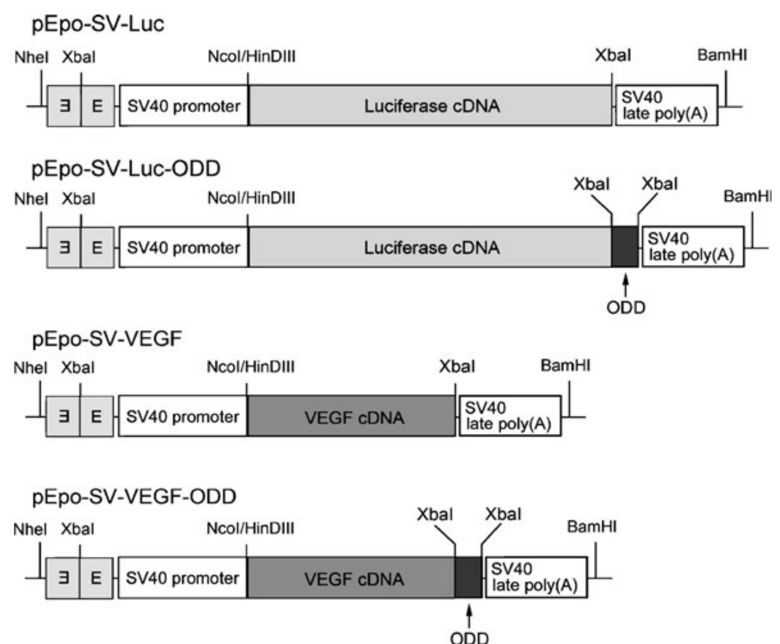

Fig. 1 Structures of the expression plasmids. The diagram shows the luciferase and VEGF constructs containing the Epo enhancer or ODD domain. E represents the Epo enhancer.

Surgical Preparation and Experimental Protocol

In vivo experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The experimental protocol was approved by the Animal Research Committee of Yonsei University College of Medicine. Eight-week-old male Sprague-Dawley rats (240±10 g) were anesthetized by intramuscular injection of ketamine hydrochloride (90 mg/kg) and xylazine hydrochloride (5 mg/kg). The anesthetized rats were incubated and ventilated with positive pressure (180 ml/min) using a Harvard ventilator (Harvard Apparatus, Milis, MA, USA). The rat heart was exposed through a 2-cm incision at the left lateral costal rib. The proximal portion of the left coronary artery was ligated with a 6–0 silk suture (Ethicon Inc., Somerville, NJ) beneath the left atrium for 1 h. After occlusion, 3 intra-myocardial injections totaling 15 μg of plasmid in 50 μl PBS solution were placed at the peripheral region of the infarct. Five days after the injection, the animals were re-anesthetized and sacrificed for VEGF assay and histological examination.

Immunohistochemical Staining

The hearts were harvested, perfusion-fixed in 10% (v/v) neutral buffered formaldehyde, and paraffin embedded. For immunohistochemical examination of human VEGF, the deparaffinized tissue sections (5 μm thick) were incubated with primary monoclonal antibody (anti-human VEGF, 1:100 dilution, AbCam, Cambridge, MA, USA), following incubation in biotinylated pan-specific universal secondary antibody. The sections were then processed by a streptavidin/peroxidase complex method using a Vectastain Universal Quick Kit (Vector Labs, Burlingame, CA, USA), followed by 3,3-diamino-benxidine (DAB, Vector Laboratories, Burlingame, CA, USA) staining. The nuclei were counterstained with methyl green. The images were distinguished at 200× magnification. To examine the formation of new micro-vessels, tissue sections (5 μm thick) were stained with anti-α-smooth muscle actin antibodies (anti-α-SMA, 1:50 dilution, AbCam, Cambridge, MA, USA) for 1 h. Texas red-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Lab, West Grove, PA, USA) was used as a secondary antibody. The staining procedures were also conducted according to the Vectastain Universal Quick Kit with DAB as substrate. The tissue sections were then counterstained with methyl green, dehydrated, and mounted with Neo-Mount® (Merck, Darmstadt, Germany). All images were made using light microscopy and processed by MetaMorph software version 4.6 (Universal Imaging Corp., Downingtown, PA, USA). Alpha-SMA-positive area was quantified as vessels per mm^2 expressing α-SMA. To calculate the average vessel number, five random fields per section were sampled.

Myocardial Infarct Size

To measure myocardial infarct size, each paraffin section was stained with Masson's trichrome. Total infarct area was measured with MetaMorph software version 4.6 and expressed as a percentage of the total left ventricle.

Terminal Deoxynucleotide Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Assay

The TUNEL assay was performed according to the manufacturer's instructions (Chemicon, Temecula, CA, USA). Briefly, the paraffin-embedded tissue sections were deparaffinized, rehydrated, and rinsed with PBS. A positive control sample was prepared from a normal heart section treated with DNase I (10 U/ml, 10 min at room temperature). The sections were pretreated with 3.0% H_2O_2, reacted with the TdT enzyme at 37°C for 1 h, and incubated with digoxigenin-conjugated nucleotide substrate at 37°C for 30 min. Nuclei exhibiting DNA fragmentation were visualized by incubation in 3,3-diaminobenzidine (DAB) for 5 min, which stained the apoptotic nuclei dark brown. The tissue sections were counterstained with methyl green, mounted, and observed under the light microscope. Four slices per group were prepared. For each sample, the cells were examined in at least 10 different regions of the slice (×400).

Statistical Analysis

One-way ANOVA was used to compare more than two groups at a time. Student's *t*-test was used to compare two groups. A *p*-value less than 0.05 was considered to indicate significance.

RESULTS

Vector Construction and *In Vitro* Transfection

To evaluate the induction of gene expression by the ODD domain in hypoxia, HEK 293 cells were transfected with pEpo-SV-Luc-ODD and pEpo-SV-Luc, and incubated further under hypoxic or normoxic conditions. When the transfected cells were exposed to hypoxic stress, luciferase expression increased (Fig. 2A). The relative level (proportional increase) of luciferase activity was greater in pEpo-SV-Luc-ODD-transfected cells than in pEpo-SV-Luc-transfected cells. The pEpo-SV-Luc and pEpo-SV-Luc-ODD constructs achieved 7-fold and 32-fold higher

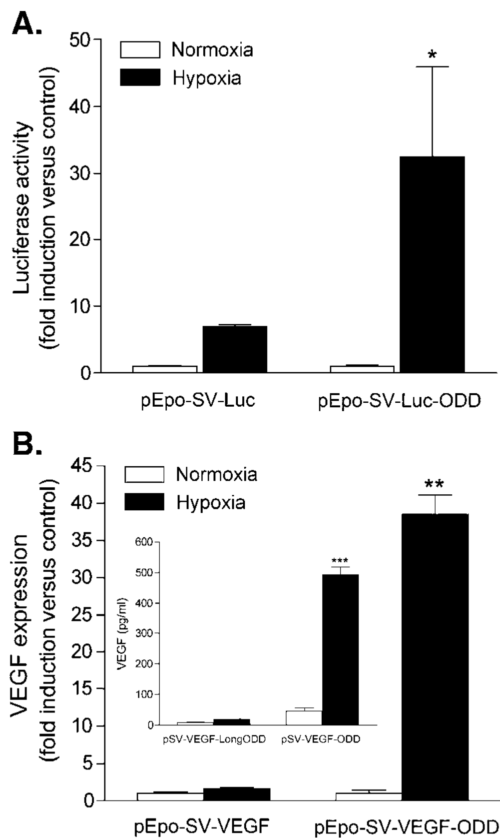

Fig. 2 Hypoxia-specific gene expression with the Epo enhancer and ODD domain. (**A**) pEpo-SV-Luc and pEpo-SV-Luc-ODD were transfected into HEK 293 cells. The control was pEpo-SV-Luc under normoxia. * denotes $P < 0.01$ as compared with normoxia. (**B**) pEpo-SV-VEGF and pEpo-SV-VEGF-ODD were transfected into HEK 293 cells. The control was pEpo-SV-VEGF under normoxia. ** denotes $P < 0.01$ as compared with normoxia. (**B Inset**) pSV-VEGF-LongODD and pSV-VEGF-ODD were transfected into HEK 293 cells. *** denotes $P < 0.01$ as compared with normoxia. The cells were further incubated under hypoxia or normoxia for 20 h after transfection. The data are expressed as mean values (±standard deviation) from four experiments.

transfection efficiency, respectively, under hypoxia than under normoxia. This result confirmed the capacity of the ODD domain combined with the Epo enhancer to augment hypoxia-sensitive gene expression. Insertion of the ODD domain into the VEGF expression vectors also enhanced the expression level of VEGF under hypoxia (Fig. 2B); in hypoxic HEK 293 cells, the pEpo-SV-VEGF-ODD construct expressed VEGF at a level about 38-fold higher than pEpo-SV-VEGF. Although the absolute values of VEGF production under hypoxia were not increased remarkably after ODD domain insertion, significant differences in VEGF expression levels between normoxia and hypoxia were observed in the ODD-containing plasmid.

The cDNA encoding the ODD domain of HIF-1α is approximately 600 bp long (22), which is much longer than that used in the present study. However, recent study has proved that a segment containing only 18 amino acids is sufficient to perform the action of ODD (26). To construct pEpo-SV-VEGF-ODD for experiments described herein, the ODD cDNA encoding these 18 amino acid residues was used. To confirm that the short ODD was effective for protein stabilization in hypoxia, pSV-VEGF-LongODD with the cDNA encoding the entire ODD domain was constructed and compared with pSV-VEGF-ODD (Fig. 2B, inset graph) by transfection of the individual constructs into HEK 293 cells. VEGF protein secreted by the transfected cells following hypoxia and normoxia were measured in the conditioned medium by ELISA. It is noted that VEGF expression increased in cells transfected with pSV-VEGF-ODD and exposed to hypoxia. This result implies that manipulating the ODD sequence encoding 18 amino acids is sufficient to stabilize the VEGF protein in hypoxia.

VEGF Expression in Ischemic Myocardium

To test the therapeutic effects of the hypoxia-inducible VEGF expression vector system *in vivo*, a rat model of myocardial ischemia was produced by ligating the anterior descending branch of the left coronary artery. After inducing myocardial infarction, pEpo-SV-VEGF and pEpo-SV-VEGF-ODD were injected into the peripheral region of the infarcted area. Five days after the injection, heart tissues and blood samples were harvested, and VEGF protein was measured by ELISA. In the pEpo-SV-VEGF-ODD group, the VEGF expression rate increased significantly more than in the pEpo-SV-VEGF and MI control groups (Fig. 3A). This result for VEGF production by pEpo-SV-VEGF-ODD *in vivo* was fully consistent with results for transfection efficiency *in vitro*. The serum samples exhibited very low levels of VEGF, which were almost undetectable by ELISA (data not shown). The result for VEGF immunohistochemical staining also showed that the VEGF production was significantly increased in the ischemic myocardium injected with pEpo-SV-VEGF-ODD, as compared to MI control and pEpo-SV-VEGF (Fig. 3B). This experiment revealed that the ODD domain can facilitate VEGF expression in hypoxia by promoting the stability of VEGF protein.

In Vivo Effects of the VEGF Expression System

To assess the anti-ischemic effects of the VEGF expression systems, the myocardial infarct size was examined by Masson's trichrome staining at 5 days after treatment (Fig. 4). Typical staining results are shown for each group in Fig. 4A. The quantification of infarct size revealed a lower degree of fibrosis in the VEGF vector treatment groups (pEpo-SV-VEGF and pEpo-SV-VEGF-ODD) than in the control (saline) group (Fig. 4B). The area of fibrosis

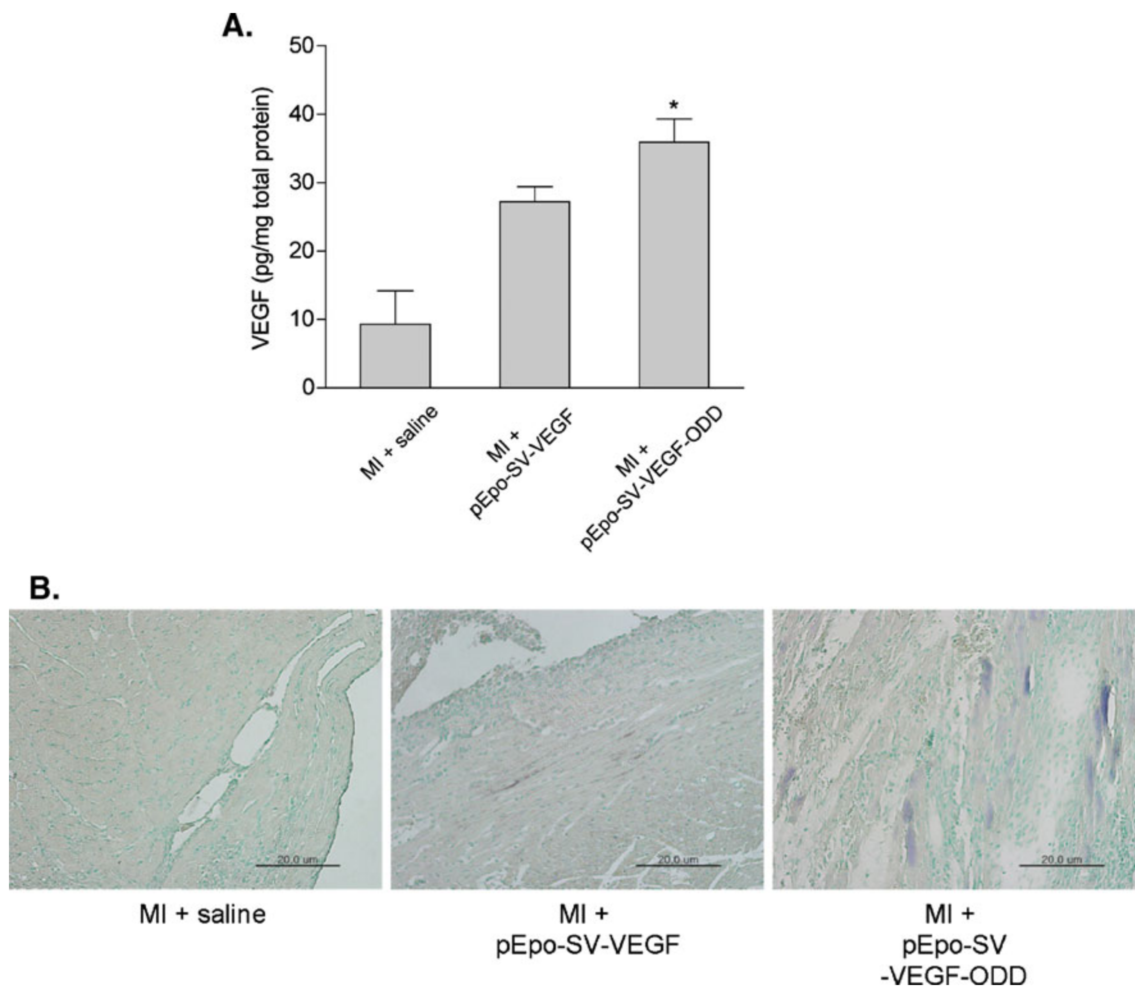

Fig. 3 Expression of VEGF in ischemic myocardium. (**A**) pEpo-SV-VEGF and pEpo-SV-VEGF-ODD were injected into rat ischemic myocardium. VEGF expression was measured by ELISA 5 days after the injection. The data are expressed as mean values (± standard deviation) from four experiments. * denotes $P<0.05$ as compared with MI+pEpo-SV-VEGF. (**B**) Immunostaining of VEGF. Five days after the injection of pEpo-SV-VEGF or pEpo-SV-VEG F-ODD, the rat myocardium was stained with anti-human VEGF antibodies. MI indicates myocardial infarction.

was remarkably reduced in the pEpo-SV-VEGF-ODD group, from 42.5% to 17% of the left ventricular wall. To investigate the effect of increased VEGF expression on neovascularization after myocardial infarction, the tissue was immunostained with anti-α-smooth muscle actin antibody (anti-α–SMA) (Fig. 5). As shown in Fig. 5A, a greater number of α-SMA-positive cells were observed in the pEpo-SV-VEGF-ODD group than in the pEpo-SV-VEGF group. Quantification of arteriole density showed that the pEpo-SV-VEGF-ODD treatment caused a 2-fold induction of micro-vessel formation relative to the MI control group, whereas pEpo-SV-VEGF increased the vessel density by only 1.2-fold (Fig. 5B). Thus, the ODD domain may accelerate post-infarct neovascularization by increasing production of VEGF proteins in their stable dimeric form.

The anti-apoptotic potential of pEpo-SV-VEGF-ODD was explored in the same rat model of myocardial ischemia using the TUNEL assay (Fig. 6). Typical results for each group are shown in Fig. 6A. The numbers of TUNEL-positive cardiomyocytes in the infarct were markedly reduced by the injection of pEpo-SV-VEGF-ODD (1.3% apoptosis), in comparison to pEpo-SV-VEGF (3.2% apoptosis) (Fig. 6B). Taken together, these results demonstrate that pEpo-SV-VEGF-ODD has a greater effect on VEGF expression in the ischemic myocardium than pEpo-SV-VEGF. This probably reflected the enhancement of VEGF expression by pEpo-SV-VEGF-ODD in hypoxia *in vivo* through the ODD-mediated protein stabilization.

DISCUSSION

In this study, we evaluated a hypoxia-inducible VEGF-ODD expression system as a gene therapy for ischemic disease, in terms of VEGF expression and histological effects. Hypoxia-specific transcriptional and translational regulatory systems have been developed using various DNA constructs (8,13). However, few studies have investigated the role of protein

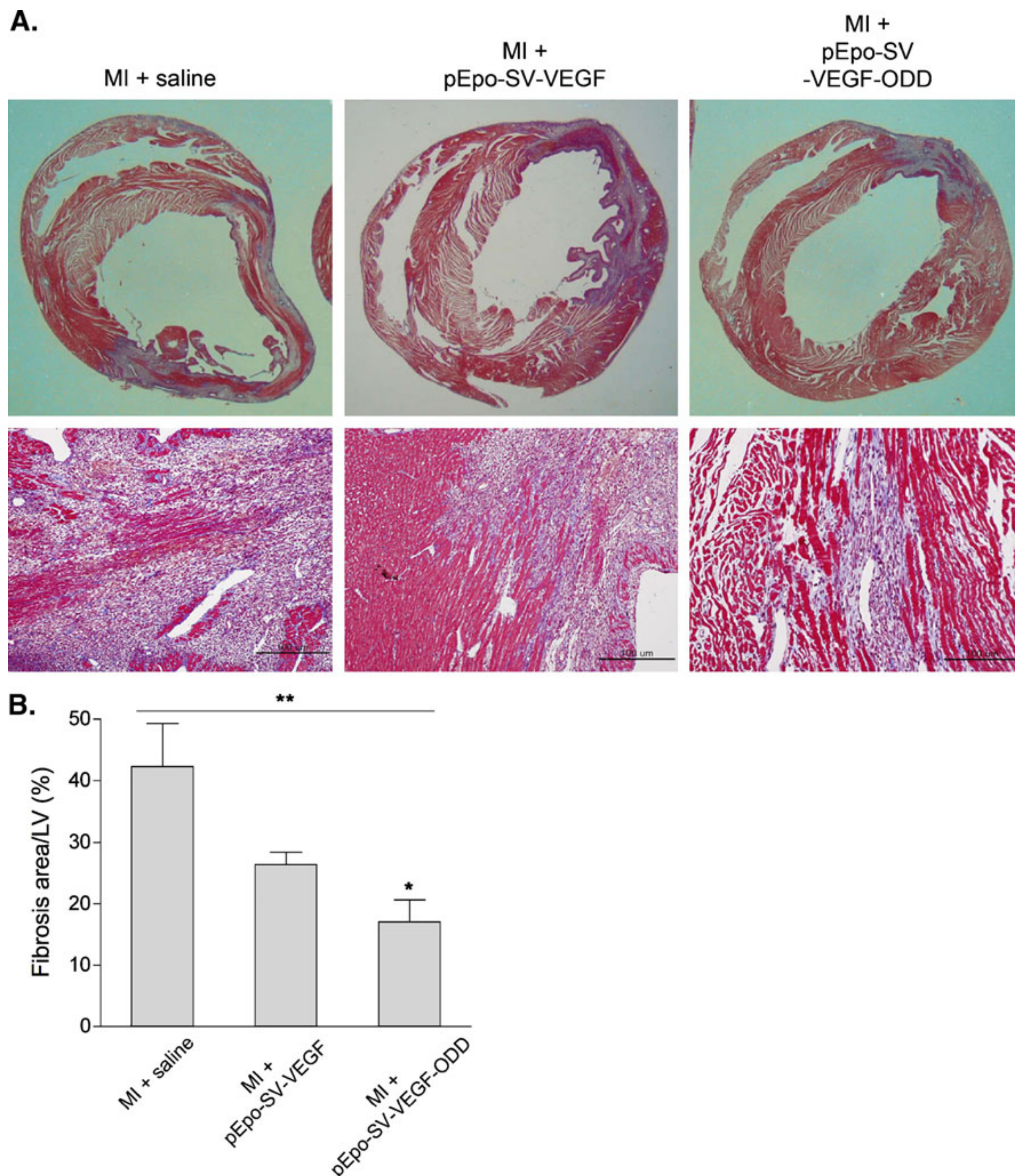

Fig. 4 Immunohistochemical staining of the ischemic heart. (**A**) Masson's trichrome staining. pEpo-SV-VEGF and pEpo-SV-VEGF-ODD were injected into the ischemic myocardium of rats. Saline was injected as a control. Fibrotic areas (collagen fibers) were stained blue with Masson's trichrome. MI indicates myocardial infarction. (**B**) Total infarct size after gene transfer was measured in the MI+saline ($n = 4$), MI+pEpo-SV-VEGF ($n = 4$), and MI+pEpo-SV-VEGF-ODD ($n = 4$) groups and expressed as a percentage of the total left ventricle. * denotes $P < 0.05$ as compared with MI+pEpo-SV-VEGF. ** denotes $P < 0.01$ as compared with MI+saline.

stabilization as a strategic element of these vector systems. For example, the ODD domain to direct hypoxia-specific expression was used to express the diphtheria toxin specifically in ischemic tumor cells (22). Another study used the ODD domain as a gene switch for hypoxia-specific heme oxygenase 1 expression (23). In a normoxic environment, presence of the ODD domain ensures that the specifically induced gene expression rapidly declines. In this way, the therapeutic increase in protein production may be precisely controlled in the regions of ischemia.

In the present study, we developed a new application for the ODD domain in ischemic disease gene therapy, i.e., to stabilize VEGF in hypoxia. We tested this application *in vitro* and *in vivo* using a construct in which the ODD

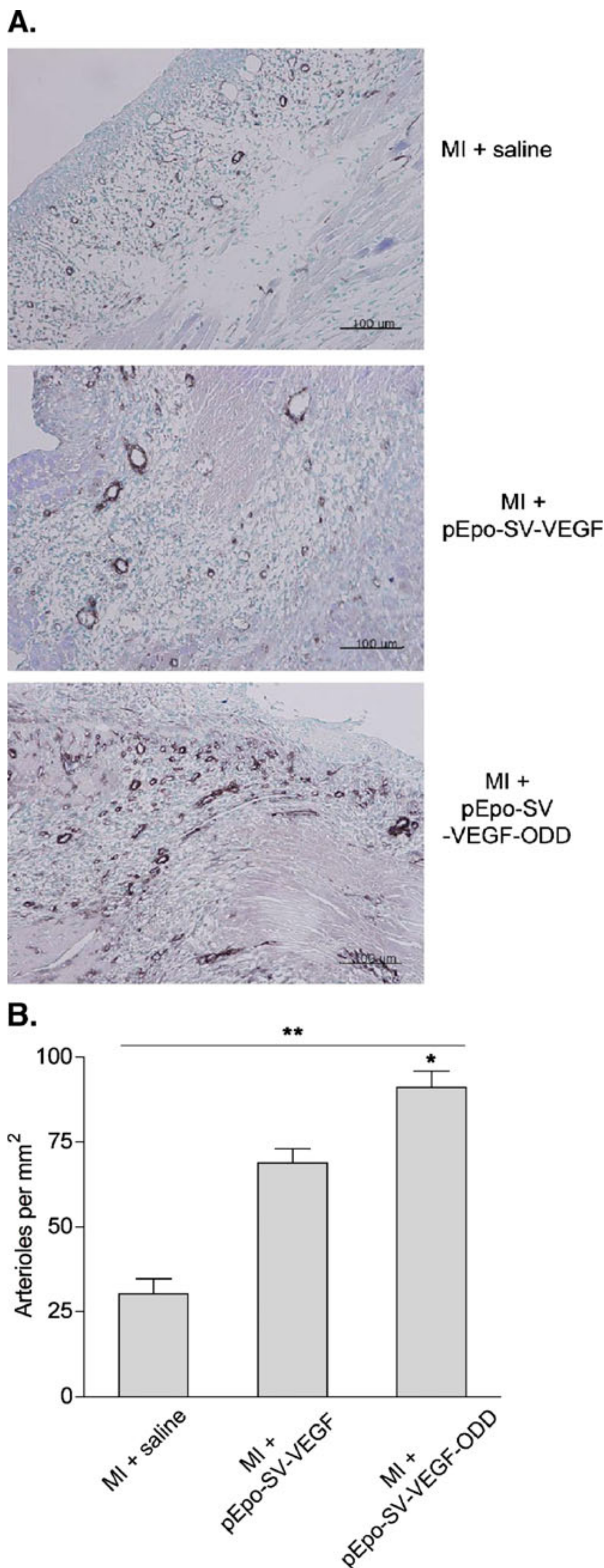

Fig. 5 Revascularization after gene transfer. (**A**) Immunostaining of α-SMA. Five days after the injection of pEpo-SV-VEGF or pEpo-SV-VEGF-ODD, the rat myocardium was stained with anti-α-SMA antibodies. MI indicates myocardial infarction. (**B**) Alpha-SMA-positive areas in the MI+ saline (n = 4), MI+pEpo-SV-VEGF (n = 4), and MI+pEpo-SV-VEGF-ODD (n = 4) groups were quantified by calculating the α-SMA positive vessels per square millimeter. * denotes $P < 0.01$ as compared with MI+pEpo-SV-VEGF. ** denotes $P < 0.001$ as compared with MI+saline.

domain was fused to the C-terminal end of VEGF (Fig. 1). During hypoxic stress, VEGF expression increased in cells (*in vitro)* and tissues (*in vivo)* treated with pEpo-SV-VEGF-ODD as compared with pEpo-SV-VEGF (Figs. 2 and 3).

Previous study revealed the efficiency of the Epo enhancer in hypoxia-inducible gene expression using HEK 293 cells exposed to hypoxia and a rabbit myocardial infarction model, respectively (8). In the present research, the ODD domain combined with the Epo enhancer affected not only transcriptional activity but also post-translational processing. We therefore assumed that this combination could more specifically regulate gene expression in response to hypoxia. Although the Epo enhancer alone increased gene transcription slightly more in hypoxia than in normoxia, the effect at this transfection level was not as large as the corresponding effect with ODD (Fig. 2). The remarkable hypoxia-dependent increase in gene expression mediated by the ODD domain (i.e., in pEpo-SV-Luc-ODD and pEpo-SV-VEGF-ODD) most likely involved both transcriptional and post-translational regulation. It is conceivable that the combination of these two regulatory strategies produced a synergistic effect on hypoxia-specific gene expression. In a previous study with pEpo-SV-VEGF-Epo-3′-UTR, the Epo enhancer and the Epo-3'-UTR were combined to regulate transcription and translation, respectively (13). The *in vitro* transfection study clearly showed that the two elements in combination can increase the specificity of gene expression in hypoxic cells. Although post-translational regulation modifies the therapeutic protein itself, the improvement in protein stability fortified the strategy for hypoxia-specific gene expression. Thus, we expected that this gene expression system combined with post-translational regulation by the ODD domain would further confine the therapeutic action to ischemic regions. In particular, we addressed the concern that the protein modification may reduce protein activity or alter normal secretion using an expression vector containing both the Epo enhancer and the ODD domain (24). Following transfection into HEK 293 cells, this construct induced hypoxia-specific gene expression to a level about 100-fold higher than that with normal oxygen. Note that the ODD domain used in the previous report was approximately 600 bp long, almost equal in size to the VEGF cDNA, which may have interfered with the folding of VEGF protein. The *in vitro* transfection assay showed that

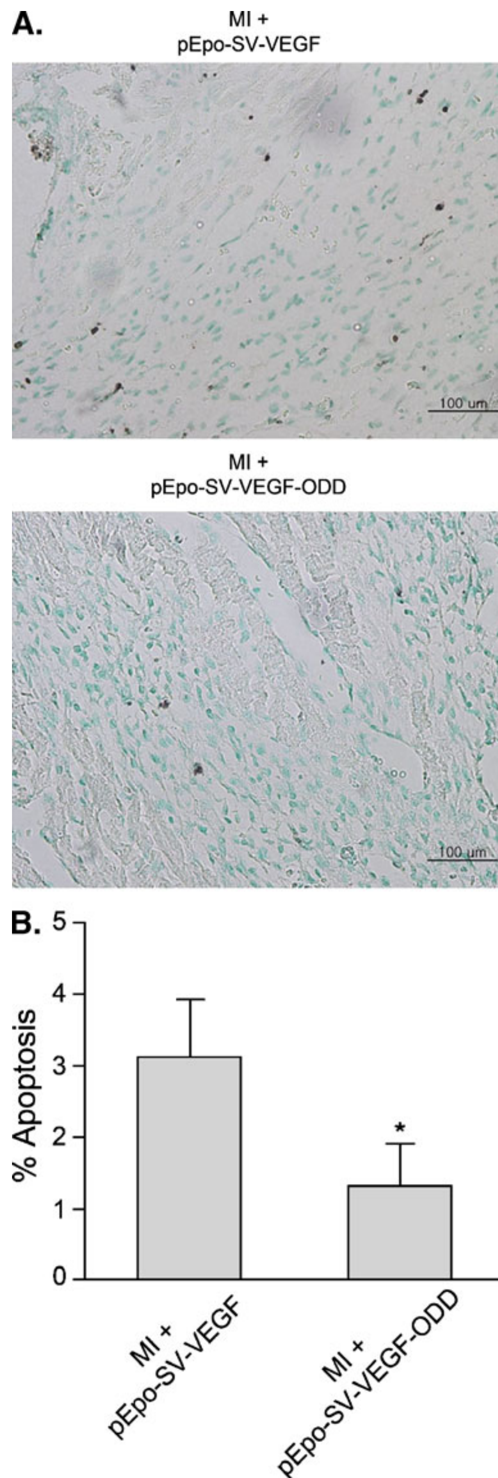

Fig. 6 Effect of pEpo-SV-VEGF-ODD on myocardial apoptosis following myocardial infarction. (**A**) Representative results of the TUNEL assay. TUNEL-positive nuclei were stained brown. Total nuclei were counted after methyl green staining. MI indicates myocardial infarction. (**B**) The level of apoptosis. The histogram shows changes in the percentage of apoptotic myocytes in each group. * denotes $P<0.05$.

the long ODD domain was not very efficient in producing active VEGF protein (Fig. 2B inset graph). Since significantly higher VEGF concentration was observed in the cytoplasm of the treated cells than in the culture medium (data not shown), the inhibitory effect on VEGF expression very likely occurred through inefficient secretion of the VEGF-ODD fusion protein. To avoid this problem herein, we inserted a cDNA encoding the short ODD domain, which contained only 18 amino acid residues, downstream of the VEGF cDNA to construct pEpo-SV-VEGF-ODD. The 18-amino acid segment was previously shown to be sufficient to constitute the active site of the ODD domain (26). In the transfection assay, the vector with the short ODD domain produced VEGF at a significantly higher level ($p<0.01$) than the vector with the long ODD domain (Fig. 2B). By *in vivo* observation, the higher VEGF expression of pEpo-SV-VEGF-ODD corresponded to greater regenerative effect in a myocardial infarct as compared to pEpo-SV-VEGF (Figs. 4, 5 and 6). Importantly, the ODD-mediated gene regulation through control of protein stability can reduce the safety concerns for VEGF gene therapy, such as disease progression in cancer patients.

CONCLUSION

A hypoxia-inducible VEGF expression vector containing the ODD domain (pEpo-SV-VEGF-ODD) was constructed and tested as a treatment for myocardial ischemia. The results *in vitro* and *in vivo* indicate that pEpo-SV-VEGF-ODD significantly increased VEGF expression through the hypoxia sensitivity of the ODD domain. Applied as gene therapy in a rat model of ischemic myocardium, pEpo-SV-VEGF-ODD reduced fibrosis and apoptosis and increased arteriole regeneration in the infarct area. This conditional control of gene expression could expand the safety profile for angiogenic gene therapy within the ischemic area. These properties of the pEpo-SV-VEGF-ODD expression vector encourage its further development as a treatment for ischemic diseases such as stroke, myocardial infarction, and peripheral artery occlusive disease. Since the inefficiency of naked plasmid DNA alone may restrict its clinical application, the use of enhancing gene delivery reagents and the development of efficient delivery systems should be further considered to significantly improve the efficiency of transgene expression *in vivo*.

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