Protection From Ischemic Heart Injury by a Vigilant Heme Oxygenase-1 Plasmid System

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Abstract—Although human heme oxygenase-1 (hHO-1) could provide a useful approach for cellular protection in the ischemic heart, constitutive overexpression of hHO-1 may lead to unwanted side effects. To avoid this, we designed a hypoxia-regulated hHO-1 gene therapy system that can be switched on and off. This vigilant plasmid system is composed of myosin light chain-2v promoter and a gene switch that is based on an oxygen-dependent degradation domain from the hypoxia-inducible factor-1-α. The vector can sense ischemia and switch on the hHO-1 gene system, specifically in the heart. In an in vivo experiment, the vigilant hHO-1 plasmid or saline was injected intramyocardially into myocardial infarction mice or sham operation mice. After gene transfer, expression of hHO-1 was only detected in the ischemic heart treated with vigilant hHO-1 plasmids. Masson trichrome staining showed significantly fewer fibrotic areas in vigilant hHO-1 plasmids-treated mice compared with saline control (43.0% ± 4.8% versus 62.5% ± 3.3%, P < 0.01). The reduction of interstitial fibrosis is accompanied by an increase in myocardial hHO-1 expression in peri-infarct border areas, concomitant with higher Bcl-2 levels and lower Bax, Bak, and caspase 3 levels in the ischemic myocardium compared with saline control. By use of a cardiac catheter, heart from vigilant hHO-1 plasmids-treated mice showed improved recovery of contractile and diastolic performance after myocardial infarction compared with saline control. This study documents the beneficial regulation and therapeutic potential of vigilant plasmid-mediated hHO-1 gene transfer. This novel gene transfer strategy can provide cardiac-specific protection from future repeated bouts of ischemic injury. (Hypertension. 2004;43:746–751.)

Key Words: ischemia • genes • heart

Myocardial ischemia associated with coronary artery disease is a leading cause of morbidity and mortality in the United States. Although percutaneous transluminal angioplasty (PTCA) and operative coronary revascularization (CABG) procedures are effective for relief of angina for most patients, there are increasing numbers of patients with severe diffuse coronary artery disease not amenable to traditional methods of revascularization. Furthermore, myocardial ischemia is asymptomatic and repeated. Thus, patients do not receive timely and proper treatments.

With our increased understanding of the key role of heme oxygenase-1 (HO-1) in the adaptation and defense against cellular stress, such as hypoxia, endotoxin, and hyperoxia, HO-1 provides a useful approach for cellular protection through anti-inflammation and antiapoptosis mechanisms. However, gene therapy strategy with HO-1 lacks the adequate spatial and temporal control. Constitutive overexpression of HO-1 may lead to cellular toxin that may, in turn, lead to kernicterus and tissue hypoxia caused by 3 catalytic byproducts of heme degradation by HO. To express the appropriate level of HO-1 to protect the heart from ischemic injury, we designed a vigilant plasmid system. The plasmid system is made of myosin light chain-2v (MLC-2v) promoter and gene switch that is based on an oxygen-dependent degradation domain (ODD) from the hypoxia-inducible factor-1-α (HIF1-α), which can sense ischemia and switch on transgene in the heart.

Previously, we have demonstrated the specificity and controlled expression of reporter gene in vitro. In this study, we hypothesized that a vigilant HO-1 plasmid system can control the expression of HO-1 gene in ischemia myocardium and protect against ischemic injury in the heart.

Methods

Construction of Vigilant HO-1 Plasmids System

The vigilant plasmid system is a double-plasmid system, which contains a sensor plasmid with the promoter and the oxygen-sensitive toggle (OST) and effector plasmid with protective gene.

In the sensor plasmid (pS-CMV-OST or pS-MLC-OST), the ODD (amino acids 394 to 603) was amplified by polymerase chain reaction from pCEP4/HIF-1α and inserted in the frame between the coding sequence of GAL4DNA binding domain and p65 activation domain (P65AD) in pS-CMV to generate pS-CMV-OST, or in pS-MLC to generate pS-MLC-OST (Figure 1). The sensor plasmid

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expresses a fusion protein (GAL4-ODD-P65AD) that binds to the effector plasmid.

The effector plasmid (pE-hHO-1) encodes human HO-1 driven by 6 copies of a 17-bp GAL4 upstream activating sequence (UAS) and an adenovirus-derived E1b TATA box. It was derived from pE/V5-His/LacZ (Invitrogen, Calif) by replacing the LacZ coding sequence with human HO-1 cDNA (a gift from Dr Agarwal, University of Florida). A 6-copy His tag was added to the C-terminal of hHO-1. The construction of the plasmids was confirmed by nucleotide sequence analysis.

**In Vitro Hypoxia Assay**

To evaluate hypoxia regulation of vigilant hHO-1 plasmids in vitro, cell transfection and hypoxic treatment were performed as described previously. HEK 293 was transfected with 1 μg pS-CMV-OST and 0.5 μg pE-hHO-1. Twenty-four hours after transfection, the medium was changed and HEK 293 cells were incubated at 0.5% or 20% O2 for 43 hours before preparation of lysates. Each condition was performed in triplicate.

**Myocardial Ischemic Model and In Vivo Gene Delivery**

In vivo experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of South Florida and were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the guidelines of the Animal Welfare Act. The experiment groups (n=20 per group) were as follows: (1) vigilant plasmid control group (V control), with intramyocardial delivery of vigilant hHO-1 plasmid with sham operation; (2) saline-treated myocardial infarction (MI) control group (S control); and (3) vigilant plasmid-treated MI group (V/MI), with intramyocardial delivery of vigilant hHO-1 plasmid with MI. Adult male BALB/c mice were subjected to a myocardial ischemia model as described previously. Briefly, male BALB/c mice were anesthetized with sodium pentobarbital (40 mg/kg, IP) and mechanically ventilated. After the heart was exposed through a lateral thoracotomy, an 8-0 polypropylene thread was passed around the left coronary artery and the artery was permanently occluded as in the MI model. Then 60 μL of vigilant hHO-1 plasmid or saline in the same volume was injected into adult BALB/c mouse hearts in the border zone surrounding the infarct 1 hour after induction of MI with a 30-gauge needle. For direct gene delivery, water-soluble polymer/plasmid complexes were prepared at a ratio of 1 μL polymer/1 μg plasmid. The plasmid complex was prepared with a fixed amount of plasmid (40 μg of pS-MLC-OST and 20 μg of pE-hHO-1) injected in a volume of 60 μL. Saline control group received an equivalent volume of a mixture of saline and polymer.

**Western Blot Analysis**

Western blot analysis was performed using 10 to 15 μg of whole cell and 125 μg of heart tissue extracts, respectively. The hHO-1/6xHis fusion protein was probed with monoclonal anti-6xHis antibody (Invitrogen, Carlsbad, Calif) and monoclonal anti-hHO-1 antibody (BD Biosciences, Palo Alto, Calif). The internal control protein paxillin was probed with antibody (BD Bioscience) for cells and GAPDH antibody (Chemicon, Temecula, Calif) for tissue, respectively. The antigen–antibody complexes were visualized by enhanced chemiluminescence (Amersham, Piscataway, NJ).

**Figure 1.** Diagram of vigilant hHO-1 plasmid system that can amplify the power of promoters based on the strong transcription activity of GAL4/p65 fusion protein. The promoter in the sensor plasmid (pS) could be replaced with CMV and MLC-2v promoter. The effector plasmid contains GAL4 upstream activation sequence (UAS) in front of an adenovirus E1b TATA box and the hHO-1/6xHis fusion gene.

**Figure 2.** A. Oxygen sensor toggle increased the expression of therapeutic gene (hHO-1) under hypoxia. After transfection with 1 μg sensor plasmid (pS-CMV-OST) and 0.5 μg effector plasmid (pE-hHO-1), 3 parallel dishes of HEK 293 cells were incubated under either 0.5% O2 or 20% O2 for 43 hours. Then cell lysates were prepared for Western blot. The hHO-1 in the effector plasmid has a 6-copy His tag. Therefore, anti-6xHis antibody was used to detect the hHO-1/6xHis fusion gene transgene without the interference of endogenous mouse HO-1. The sample taken from 3 parallel dishes were labeled as 1, 2, and 3. Paxillin is served as control for loading volume. B. Quantitation of the protein level of hHO-1/6xHis fusion protein with Western blot (P<0.01 between hypoxic-treated group and normoxic group).

**Figure 3.** Effect of myocardial ischemia on hypoxia-induced hHO-1 protein expression in mouse heart at 10 minutes, 30 minutes, 24 hours, 48 hours, 72 hours, 7 days, and 10 days after gene transfer in ischemic heart. After left ventricular ischemia, hearts were analyzed for hHO-1 using the hHO-1 antibody and 6xHis antibody, separately. The results show a higher density of blots for up to 7 days compared with endogenous levels, as shown in the control (ischemia without vigilant hHO-1 plasmid; GAPDH is shown to confirm integrity and equal loading of protein).
Histology and Immunohistochemical Analysis
At 10 days after plasmid injection, 6 hearts from 3 groups were excised. Heart was cut horizontally at the level of the papillary muscle. A horizontal section stained with Masson trichrome at the papillary muscle level was used to evaluate the histology and fibrotic deposition in the mouse heart. Expression of hHO-1 and apoptosis protein in the heart was detected by immunohistochemistry using DAKO ARK Kit according to the protocol. Mouse anti-human HO-1 (BD Bioscience) was used to detect gene expression; mouse anti-Bcl-2, rabbit anti-Bax, rabbit anti-Bak, and rabbit anti-caspase 3 (Upstate, Lake Placid, NY) were used additionally to determine the apoptosis regulator. The area of fibrotic tissue in the cross section of hearts was measured by the computer software (Scion image) after Masson trichrome staining or immunohistochemistry.

Left Ventricular Hemodynamic Assessment
After 10 days of treatment, animals were anesthetized by pentobarbital (40 mg/kg) intraperitoneally. The abdomen was opened in the middle line; a 27-gauge needle connected with pressure transducer was punched into the left ventricle chamber through the diaphragm. The left ventricular pressure was digitized using the commercially available data acquisition system (PowerLab, ADInstruments). After steady-state had been established, left ventricular developed pressures (LVDPs) and maximal rise and fall rates of pressure (\( \pm dP/dt \)) were recorded in the closed-chest preparation.

Statistical Analysis
Results are presented as means±SD. Significance between 2 measurements was determined by Student \( t \) test. Values of \( P<0.05 \) were considered significant.

Results

Hypoxia Switches on Vigilant hHO-1 Plasmid System In Vitro
HEK 293 cells were transfected with sensor plasmid pS-OST and effector plasmid pE-hHO-1-\( \alpha \), and then subjected to 0.5% \( \text{O}_2 \) for 43 hours. The hHO-1 expression regulated by the OST construct increased by 12.6-fold in cells incubated under hypoxia relative to cells incubated under 20% \( \text{O}_2 \). Thus, ODD-based double plasmid system show significant hypoxic induction (Figure 2a, b).

Myocardial Ischemia Switches on Vigilant hHO-1 Plasmid System In Vivo
Western blot analysis of heart homogenates showed that time of onset of vigilant plasmid system after ischemia is \( \sim 10 \) minutes in myocardium. As shown in Figure 3, myocardial ischemia caused a time-dependent increase in hHO-1 protein (32 kDa) expression, keeping high levels from 24 hours until day 7. The timeframe is in keeping with the clinically accepted timeframe of 2 to 6 hours for therapeutic intervention. It demonstrated that hypoxia in ischemic myocardium activates the OST and subsequently drives hHO-1 expression. Antibody for 6xHis tag identifies hHO-1/6xHis fusion protein and distinguishes human HO-1 expression from hypoxia-mediated endogenous mouse HO-1 expression (Figure 3).

Ten days after gene transfer, immunohistochemical detection revealed intense staining corresponding to HO-1 immunoreactivity in the peri-infarct zone of hHO-1-vigilant plasmid-treated hearts but not in sections prepared from sham-operated hearts treated with the same plasmid.

Intramyocardial Delivery of Vigilant hHO-1 Plasmids Provides Antiapoptosis Protection and Reduces Intersitial Fibrosis
Ten days after gene transfer, Masson trichrome staining showed significantly fewer fibrotic areas in vigilant hHO-1 plasmid-treated mice compared with saline control (43.0%±4.8% versus 62.5%±3.3%, \( P<0.01 \)) (Figure 5a). The reduction of interstitial fibrosis is accompanied by an increase in myocardial hHO-1 expression in peri-infarct border areas. Morphometric analysis of serial sections showed that antiapoptotic Bcl-2 expression was increased and proapoptotic Bax, Bak, and caspase 3 expression was decreased in the peri-infarct area of hHO-1-vigilant plasmid-transfected hearts than in saline-injected control hearts (Figure 5b).

Intramyocardial Delivery of Vigilant hHO-1 Plasmids Improves Left Ventricular Function
To determine whether vigilant hHO-1 plasmid system protects against postischemic injury, we used cardiac catheter to compare LVDP (the difference between left ventricle systolic pressure and left ventricle end-diastolic pressure, mm Hg) and the maximum and minimum values of the first derivative of left ventricle pressure (\( +dP/dt \) and \( -dP/dt \), mm Hg/s) between vigilant hHO-1 plasmid-treated MI group and saline-treated MI group. At 10 days after gene transfer, vigilant hHO-1 plasmid-treated mice had higher LVDP than did the
heart from saline control (104.2±9.9 versus 77.5±6.5, 
\( n=6; \ P<0.01 \)). Vigilant hHO-1 plasmid-treated mice also showed improved systolic functional recovery, as demonstrated by higher +dP/dt compared with that in saline control mice (2605.3±144.2 versus 1819.8±80.3 mm Hg/s, \( n=6; \ P<0.01 \)). Simultaneously, the −dP/dt was significantly higher in the vigilant hHO-1 plasmid-treated animals than in saline-control animals (−1969.3±90.9 versus −1762.7±145.9 mm Hg/s, \( n=6; \ P<0.01 \)), indicating better diastolic function (Figure 6).

**Figure 5.** Histology of the ischemic heart on day 10. A, Masson trichrome-stained transverse sections at the papillary muscle level of infarcted mouse hearts. Thinning of the infarcted left ventricular wall can be seen. B, Immunohistochemical staining for Bcl-2, Bax, Bak, and caspase 3 proteins (original ×200) in ischemic myocardium of vigilant hHO-1 plasmid-treated group and saline-treated group on day 10. Masson trichrome staining showed significantly fewer fibrotic areas in vigilant hHO-1 plasmid-treated mice compared with saline control (43.0%±4.8% versus 62.55±3.3%, \( P<0.01 \)). The reduction of interstitial fibrosis is accompanied by an increase in myocardial hHO-1 expression in peri-infarct border areas, concomitant with higher Bcl-2 levels and lower Bax, Bak, and caspase 3 levels in the ischemic myocardium compared with saline control.

**Discussion**

The results show that a hypoxia-sensitive vigilant plasmid expresses hHO-1 in mice with myocardial ischemia and provides cardioprotection. The cardioprotection is caused by the HO-1-inducing apoptosis during prolonged hypoxia.

An endogenous cellular defensive system\(^8\) is initiated by hypoxia to activate genes that protect against cellular injury.\(^9\)–\(^13\) These include heat shock protein,\(^14\)–\(^15\) vascular growth factor,\(^16\)–\(^18\) among others, which have been tested in ischemic models. However, endogenous expression of protective genes...
is not strong enough. Adding recombinant genes increases their effectiveness, especially for long-term myocardial protection. Melo et al introduced recombinant adeno-associated virus-mediated delivery of HO-1 gene into ischemic rat hearts and showed an effective decrease in myocardial apoptosis and inflammation. Vulapalli et al generated α-MHC promoter/HO-1 transgenic mice for cardioselective overexpression of HO-1 and showed that selective expression of HO-1 in ischemic hearts is cardioprotective via an anti-apoptotic action. For gene therapy for human ischemic heart disease, we need further development of these strategies. For example, there are concerns that in treatment of human ischemic heart disease with long-term overexpression of HO-1, there will be toxic or malignant effects. Furthermore, the majority of MI is asymptomatic, and repeated bouts of myocardial ischemia cause accumulative cardiac tissue damage. Therefore, what patients need is a gene therapy strategy that acts in the heart and switches on or off, so that the therapeutic protein would be produced only where and when it is needed.

We have developed a strategy to use pathophysiological signals to switch on transgenes and amplify their expression, which we call “vigilant vector.” Recently, several studies have attempted to develop hypoxia regulated under the control of hypoxia response element promoters and have demonstrated that hypoxia response element promoters regulated vascular growth factor secretion in vitro and in MI animal models. However, this system is not ideal for treatment of ischemic heart disease because of the low efficiency of cardiac-specific gene expression in response to hypoxia loads. An amplification or overexpression of cardioprotective genes is necessary during acute MI. We have developed a novel double-plasmid approach based on the ODD of HIF-1α by separating the promoter and ODD into a sensor plasmid and the transgene into an effector plasmid. Under normal oxygen conditions, the ODD is essentially undetectable caused by its rapid destruction by the ubiquitin-proteosome system. During hypoxia, ODD is not degraded and accumulates exponentially as cellular O₂ decreases. The ODD therefore acts as a gene switch that is on during hypoxia and does not activate endogenous genes. The sensor plasmid expresses a fusion protein (Gal4 ODD P65), which binds to the 6 copies of GAL4 UAS in the effector plasmid. In vivo data showed that the double-plasmid method dramatically increased the power of the cardiac selective promoter (MLC-2v) by more than 300-fold. Therefore, MLC-2v was used as the promoter to drive the sensor plasmid gene chimaera (Gal4 ODD P65).

We cotransfected the sensor plasmid and effector plasmid in ischemic mouse myocardium and demonstrated that the vector in cardiomyocytes responded to pathophysiologic levels of the oxygen level in vivo. The effector plasmid expressed HO-1 with response to myocardial ischemia and promptly expressed hHO-1 for 1 week in ischemic myocardium. The use of hHO-1 with 6xHis attached differentiated the expression of the plasmids from expression of endogenous mouse HO-1 genes. The results show a highly amplified signal (>12-fold) with the vigilant plasmid.

The effect of hHO-1 was on inhibiting apoptosis. Apoptosis of cardiomyocytes is initiated by a number of cell death triggers that stimulate the release of several proteins. Bcl-2 is an antiapoptotic protein that regulates a cell’s predisposition to undergo apoptosis through sequestration of critical activation factors or alterations of mitochondrial permeability, whereas, Bax, Bak, and caspase 3 are proapoptotic proteins. In our study, we found that Bcl-2 is upregulated in myocardium treated with vigilant hHO-1 plasmids compared with myocardium treated with saline. Moreover, the Bax and caspase expressions were downregulated in vigilant plasmid-treated myocardium compared with saline-treated myocardium, reflecting that hHO-1 regulates acute myocyte loss in ischemic myocardium through reducing apoptosis. Taken together, these findings support an important role of HO-1, which is decreasing apoptosis for prevention of acute ischemic damage of heart that contributes to preservation of cardiac function.

In summary, we provide direct evidence that a vigilant plasmid system can be activated in vivo in ischemic myocardium and protects ischemic hearts from acute damage. However, the plasmid system is only fit for short-term protection (7 days) because of transient expression of plasmids. To provide long-term protection of the heart during ischemia, we need to construct these components into a recombinant adeno-associated viral plasmid to make a viral vigilant system. Our data and results from other studies show...
that a single injection of rAAV (serotype 2) expresses transgenes in tissue that can last for at least 6 to 18 months.22

Perspectives

In summary, the studies expand current gene therapy that simply overexpresses therapeutic genes constitutively. We have developed a vigilant plasmid system that is responsive to physiological regulators, such as oxygen level, so that cardioprotective genes can be switched on or off. We also demonstrate the ability to regulate HO-1 expression in the heart by a selective promoter. This opens the potential for a gene therapy approach to patients at high risk for coronary artery disease, because the risk of a second heart attack is very high. We envision a therapy in which the vigilant vector could be administered after the first heart attack.

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