Gene Therapy for Myocardial Protection

Transfection of Donor Hearts With Heat Shock Protein 70 Gene Protects Cardiac Function Against Ischemia-Reperfusion Injury

Jay Jayakumar, MD; Ken Suzuki, MD, PhD; Mak Khan, PhD; Ryszard T. Smolenski, MD, PhD; Aldo Farrell, PhD; Najma Latif, PhD; Olivier Raisky, MD; Haitham Abunasra, MD; Ivan A. Sammut, PhD; Bari Murtuza, MD; Mohamed Amrani, MD, PhD; Magdi H. Yacoub, DSc, FRS

Background—Heat shock protein 70 (HSP70) gene transfection has been shown to enhance myocardial tolerance after normothermic ischemia-reperfusion. We investigated the effect of HSP70 gene transfection on mechanical and endothelial function in a protocol mimicking clinical heart preservation.

Methods and Results—Rat hearts were infused ex vivo with Hemagglutinating Virus of Japan–liposome complex containing HSP70 gene (HSP, n=8) or no gene (CON, n=8), and heterotopically transplanted into recipient rats. Four days after surgery, transfected hearts were perfused on a Langendorff apparatus for 45 minutes, arrested with St Thomas’ No. 1 cardioplegia for 4 hours at 4°C, and reperfused for 1 hour. Mechanical and endothelial function was studied before and after ischemia. Creatine kinase was measured in reperfusion effluent. Hearts underwent Western blotting and immunohistochemistry to confirm HSP70 overexpression. Postischemic recovery of mechanical function (% preischemic±SEM) was greater in HSP versus CON: Left ventricular developed pressure recovery was 76.7±3.9% versus 60.5±3.1% (P<0.05); dP/dtmax recovery was 79.4±4.9% versus 56.2±3.2% (P<0.05); dp/dtmin recovery was 74.8±4.6% versus 57.3±3.6% (P<0.05). Creatine kinase release was attenuated in HSP versus CON: 0.22±0.02 versus 0.32±0.04 IU/min/g wet wt. (P<0.05). Recovery of coronary flow was greater in HSP versus CON: 76.5±3.8% versus 59.2±3.2% (P<0.05). Recovery of coronary response to 5-hydroxytryptamine (5×10−5 mol/L) was 55.6±4.7% versus 23.9±3.2% (P<0.05); recovery of coronary response to glyceryltrinitrate (15 mg/L) was not different between HSP and CON: 87.4±6.9% versus 84.3±5.8% (NS).

Conclusions—In a clinically relevant donor heart preservation protocol, HSP70 gene transfection protects both mechanical and endothelial function. (Circulation. 2000;102[suppl III]:III-302-III-306.)

Key Words: genes ■ proteins ■ ischemia ■ reperfusion ■ transplantation

Heat shock proteins (HSPs) are a family of inducible and constitutively expressed intracellular proteins that have a major role in protecting cells from the effects of environmental stress. They act by playing an essential role in protein folding and translocation and as chaperones for intracellular proteins. The levels of HSPs are increased by a variety of environmental stresses, including heat stress and ischemia-reperfusion injury.

Previous work has shown that a rise in levels of a particular 70-kDa HSP (HSP70), induced by heat stress, is associated with protection against ischemia-reperfusion injury. We have shown improved recovery of both ventricular and coronary endothelial function of rat hearts after heat stress, in a protocol involving prolonged cardioplegic arrest and reperfusion.

The mechanisms by which heat stress leads to protection of ventricular and endothelial function after ischemia-reperfusion injury may involve many pathways. These include not only an increase in HSP70 levels but also induction of free-radical scavengers and attenuation of apoptosis. Recent studies from our laboratory have also indicated a role for beneficial changes in metabolic pathways after heat stress, in protocols involving normothermic and hypothermic ischemia.

To study the role of individual HSPs rather than the many complex pathways induced by heat stress, techniques available include the use of transgenic animals overexpressing HSPs and gene transfection. We used an established in vivo gene transfection technique, which has been shown to provide high-level expression of protein in the whole heart, with intracoronary infusion of Hemagglutinating Virus of Japan (HVJ)-liposome complex to transfect rat hearts with the gene for HSP70.

In a clinically relevant model of donor heart preservation involving cardioplegic arrest, prolonged hypothermic ischemia, and reperfusion, we investigated if HSP70 gene transfection in a rat cardiac transplant model leads to preservation of ventricular and endothelial function.
Methods

Animals
Male Sprague-Dawley rats were used in all studies; donor rats weighed 225 to 250 g, and recipient rats weighed 325 to 350 g. Animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research; the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources (NIH publication No. 86-23, revised 1985); and the European Convention on Animal Care guide. The study was approved by the institutional ethics committee on animal research.

Construction of Expression Vector
Full-length human HSP70 cDNA12 (donated by Dr S. Fox and Dr R. Morimoto, Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Ill) was cloned at the EcoRI/BamHI site of pcDNA3, which has a cytomegalovirus promoter (Invitrogen Corp).11

Preparation of HVJ-Liposome Complex
The preparation of the HVJ-liposome complex (donated by Prof Y. Kaneda, Osaka University, Japan) has been described previously.13 Briefly, 10 μg of lipid mixture (phosphatidylserine, phosphatidylcholine, and cholesterol) was deposited on the side of a flask by removing tetrahydrofuran in a rotary evaporator. The dried lipid was hydrated in 200 μL of balanced salt solution (137.0 mmol/L NaCl, 5.4 mmol/L KCl, 10.0 mmol/L Tris-HCl; pH 7.6) containing a DNA (200 μg)-HMG1 (high-mobility group 1 nuclear protein, 64 μg) complex.

A liposome-DNA-HMG1 complex suspension was prepared by vortexing, sonication, and shaking to form liposome. The liposome complex.

Gene Transfection
Gene transfection was performed on hearts of Sprague-Dawley rats (225 to 250 g), as described previously.14 Donor rats were anesthetized with sodium pentobarbital (50 mg/kg), and sodium heparin (1000 IU/kg) was injected through the femoral vein. Their hearts were arrested with cold cardioplegia injected retrograde through the abdominal aorta [St Thomas’ Hospital cardioplegic solution No. 1, supplied as a concentrate (Martindale), was diluted (1:50) in Ring-
er’s solution (Travenol Labs) and filtered].

A thoracotomy was performed, and hearts were excised. Hearts from the group transfected with the HSP70 gene (HSP, n=8) were infused with 1 mL of HVJ-liposome containing pcDNA3 with human HSP70 cDNA through the coronary artery, with the venae cavae, pulmonary arteries, and veins ligated. The control hearts (CON, n=8) were infused with the same volume of HVJ-liposome containing pcDNA3 but without the HSP70 gene. After incubation on ice for 10 minutes, the hearts were then heterotopically trans-plantated into the abdomens of recipient rats (300 to 325 g) of the same strain.15

Recipient rats were killed on the fourth day after gene transfection, thus allowing the introduced gene to express proteins stably and providing adequate time for intrinsic HSP70 induced by surgical stress to decrease to preoperative levels.11

Functional Assessment
HSP70 and control gene-transfected hearts were studied to determine ventricular and endothelial function before, during, and after 4 hours of cardioplegic arrest at 4°C (Figure 1).

Rats were anesthetized with diethyl ether, and sodium heparin (1000 IU/kg) was injected through the femoral vein. Transplanted hearts were rapidly excised, placed in ice-cold Krebs-Henseleit buffer, immediately attached to a Langendorf apparatus, and perfused with filtered Krebs-Henseleit buffer (118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO4, 1.2 mmol/L KH2PO4, 24 mmol/L NaHCO3, 11 mmol/L glucose, 1.2 mmol/L CaCl2, pH 7.4) at a constant pressure of 100 cm H2O and continuously gassed with a 95% O2/5% CO2 mixture at 37°C, as described previously.10

After an initial stabilization period of 20 minutes of normoxic perfusion, preischemic mechanical function was evaluated with the use of an intraventricular balloon. Subsequently, hearts were arrested by infusion of 4°C cardioplegia at a constant pressure of 60 cm H2O for 2 minutes. Hearts were immersed in cardioplegia and maintained at 4°C with the aid of a temperature probe.

After 4 hours of cardioplegic arrest, hearts were reperfused with Krebs-Henseleit buffer at 37°C, and coronary effluent was collected during the first 15 minutes of reperfusion. After 30 minutes of reperfusion, postischemic mechanical function was evaluated. At the end of the experiments, hearts were freeze-clamped in liquid nitrogen for Western blot analysis.

Hearts were not paced during the entire protocol; preischemic and postischemic heart rates were recorded after 30 minutes of perfusion and 30 minutes of reperfusion, respectively.

Ventricular Function
Ventricular function was assessed with a balloon catheter inserted into the left ventricle, as previously described.16 The balloon was inflated to an end-diastolic pressure of 10 mm Hg. Peak systolic pressures were recorded and used to calculate developed pressure. Recovery of mechanical function was expressed as relative recovery of postischemic versus preischemic developed pressure (relative recovery of developed pressure) and time derivatives of pressure changes (+dP/dt and −dP/dt).

Endothelial Function
Coronary flow was recorded with an electromagnetic flowmeter (Scalar). Endothelial function was assessed through observations of preischemic and postischemic coronary flow responses to 5-hydroxytryptamine (5-HT) (10−5 mmol/L, 10−4 mmol/L, 10−3 mmol/L) and glyceryltriminitrate (GTN) (15 mg/L). For final calculations, the response to 10−2 mmol/L 5-HT was used. Our protocol for this test has been described in earlier studies.17 In the intact endothelium, 5-HT causes vasodilation through the release of endothelium-derived...
relaxing factor, whereas in the presence of endothelial damage, it causes vasoconstriction by a direct effect on smooth muscle. GTN causes vasodilation by an endothelial-independent effect on smooth muscle.

**Western Blotting**

HSP70 concentration was assessed at the end of reperfusion in both groups (n=5/group) by Western immunoblotting, as previously described. Whole-heart homogenates were solubilized in 1% wt/ vol SDS, assayed for total protein with the Bradford assay, denatured by heating at 100°C in Laemmli buffer, and separated on 10% SDS gels until the bromophenol blue tracking dye reached the end of the gel. The gels were equilibrated for 30 minutes in transfer buffer before protein transfer at 500 mA for 1 hour. Western blots were blocked for 1 hour with 3% wt/vol skimmed milk powder (Marvel) in PBS (0.15 mol/L NaCl, 0.05 mol/L phosphate buffer, pH 7.2) containing 0.05% wt/vol Tween-20; this blocks nonspecific binding sites. Blots were then probed with monoclonal mouse antibody to inducible HSP70 (SPA-810; Stress Gen Biotechnologies Corp) diluted to a final concentration of 1:1000, for 1 hour. Blots were washed 3 times and incubated with secondary horseradish peroxidase–conjugated rabbit anti-mouse antibody for 1 hour.

Blots were visualized with the use of an enhanced chemiluminescence (ECL) detection system (Amersham). Hyperfilm MP (myoperoxidase) was exposed to blots treated with ECL for 30 seconds and developed in an automatic film processor; after ECL exposure, fluorescence (ECL) detection system (Amersham). Hyperfilm MP (myoperoxidase) was exposed to blots treated with ECL for 30 seconds and developed in an automatic film processor; after ECL exposure, ECL films were scanned with a Molecular Dynamics 300A laser densitometer, and HSP70 levels were determined as a proportion of total protein loaded with the use of Quantity One software (PDI).

**Immunohistochemistry**

Hearts from both groups (n=3 from each group) were removed from the Langendorff apparatus at the end of reperfusion and quickly divided into 2 parts. One part was immediately frozen in embedding medium, OCT compound (Miles Inc, Diagnostics Division) with liquid nitrogen. The samples were cut into thin sections (5 μm). After blocking with 5% FBS, the sections were incubated first with a 1:1000 dilution of monoclonal mouse antibody to inducible HSP70 (SPA-810) followed by incubation with a 1:180 dilution of FITC-conjugated goat anti-mouse IgG monoclonal antibody. The sections were observed with a fluorescence microscope. Immunohistochemical analysis was also performed on additional hearts 4 days after transfection (HSP, n=3; CON, n=3), which did not undergo perfusion, to compare the effects of perfusion on HSP expression.

**Statistics**

Values are presented as mean±SEM. Statistical comparison was performed by an unpaired Student’s t test. A value of P<0.05 was considered a significant difference.

**Results**

Western blot analysis indicated stronger expression of HSP70 in HSP versus CON (Figure 2). Tubulin expression was constant throughout the lanes, indicating equal protein loading on the blot. According to semiquantitative analysis with computed densitometry, the mean levels of HSP70 in HSP and CON were 4.12±0.55 (range 1.11 to 6.44) and 0.60±0.54 (range 0.02 to 4.93), respectively. This represented nearly a 7-fold increase in HSP70 expression in HSP versus CON (P<0.05).

**Immunohistochemistry**

Immunohistochemical examination showed apparent and extensive overexpression of HSP70 in the cytoplasm of cardiomyocytes in HSP as well as cytoplasm of coronary endothelial cells, as compared with those from CON. Approximately 60% of the cardiomyocytes in hearts from HSP were shown to overexpress HSP70.

Also, there were no appreciable differences in immunostaining between the perfused and nonperfused hearts from both HSP and CON. These results correlated well with previous immunohistochemical studies in a similar protocol of HSP70 or sham gene transfection and subsequent perfusion.

**Ventricular Function**

Postischemic recovery of mechanical function (%preischemic baseline mean values±SEM; at 10 mm Hg left ventricular end-diastolic pressure) was greater in HSP versus CON (Figure 3). Left ventricular developed pressure recovery was significantly higher in HSP versus CON: 76.7±3.9% versus 60.5±3.1% (P<0.05). The maximum dP/dt recovery was significantly higher in HSP versus CON: 79.4±4.9% versus 56.2±3.2% (P<0.05); likewise the minimum dP/dt recovery was also significantly higher in HSP versus CON: 74.8±4.6% versus 57.3±3.6% (P<0.05).

Heart rates were not significantly different between HSP versus CON after 30 minutes of perfusion: 254±11 versus 248±9 bpm (NS), nor after 30 minutes of reperfusion.
various noxious stimuli, including thermal stress 21 and ische-
the beneficial effects of HSP70 gene transfection against
preservation protocols. Previous work has also demonstrated
after cardioplegic arrest, which mimics clinical donor heart
protocol used a prolonged period of hypothermic ischemia
period of ischemia and reperfusion; however, endothelial
function was not formally assessed. Also, these protocols
transfected hearts compared with control gene–transfected
chemic recovery of mechanical function in HSP70 gene–
This study demonstrated significant improvement of postis-
function in HSP70 gene–
advantages of the present protocol include use of a highly
efficient in vivo gene transfection technique and established
methods for assessment of ventricular and endothelial function.
Limitations of our protocol include the use of rats and the
use of crystalloid fluid for perfusion. HSP70 mRNA levels have
been shown to increase in Langendorff-perfused hearts
in comparison with unperfused hearts;24 however, total
HSP70 levels remained similar because of the longer period
required for protein synthesis. Comparison of immunohisto-
chemistry results from perfused and nonperfused gene-
transfected hearts revealed no significant difference in HSP70
expression; thus, we conclude that Langendorff perfusion
does not significantly alter HSP70 levels.

Gene transfection 4 days before assessment of cardiac function
was required in our protocol. This was partly designed to reduce
the transplant-associated HSP70 induction in CON (stress-induced rise in HSP70 levels return to
prestress levels by 4 days).18 Furthermore, this interval allows
optimal level of HSP70 expression resulting from gene
transfection.11 Thus, our protocol is not directly applicable
to the clinical situation; nevertheless, it provides a reliable
experimental model for investigating gene therapy for myocardial
protection. Advances in transfection techniques may
allow a more rapid induction of protein expression, especially
if genes can be introduced into the heart by catheter tech-
niques before organ donation.24 Furthermore, patients with
high initial myocardial levels of inducible HSP70 had better
cardioprotection during cardiac surgery; heat shock proteins
could thus have a role in clinical gene therapy for myocardial
protection.25

In summary, this study demonstrates improved preservation
of ventricular and endothelial function in HSP70 gene–
transfected hearts, in a protocol mimicking conditions for
heart preservation; gene therapy may provide a novel ap-
proach for myocardial protection in the setting of clinical
transplantation.

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