Gene Transfer of Heat-Shock Protein 70 Reduces Infarct Size In Vivo After Ischemia/Reperfusion in the Rabbit Heart

Shinji Okubo, MD, PhD; Oliver Wildner, MD; Maulik R. Shah, MD, PhD; Jeya C. Chelliah, PhD; Michael L. Hess, MD; Rakesh C. Kukreja, PhD

Background—Heat-shock protein 70 (HSP 70) plays a role in myocardial protection. No studies are available, however, to show that direct gene transfer of HSP 70 reduces myocardial infarction in vivo.

Methods and Results—Rabbit hearts were injected with vehicle or Ad.HSP70 at 3 sites (1.5×10⁹ pfu, 50 μL/site) in the left ventricle (LV). Four days later, hearts were removed, and expression of inducible (HSP 70) and constitutive (HSC 70) proteins was measured in the LV and right ventricle (RV). Subsets of 5 to 7 animals in the vehicle-, Ad.lacZ-, and Ad.HSP70-treated groups were subjected to 30 minutes of ischemia and 3 hours of reperfusion. Infarct size was measured by tetrazolium staining. Increased expression of HSP 70 was observed in LV injected with Ad.HSP70 compared with vehicle-treated hearts. HSP 70 was undetectable in RV, the noninjected region of the heart. The expression of HSC 70 remained unchanged in hearts treated with vehicle or Ad.HSP70. Infarct size (% risk area) decreased to 24.5±2.8 in Ad.HSP70-injected hearts compared with 41.9±2.8 and 42.7±2.5 in the vehicle- and Ad.lacZ-treated hearts (P<0.01). The infarct size was not different between the vehicle- and Ad.lacZ-treated hearts (P>0.05). The risk areas (% of LV) were not different among the 3 groups, ie, 50.1±5.2, 47.7±3.5, and 53.3±2.9 in vehicle-, Ad.lacZ-, and Ad.HSP70-treated groups (P>0.05).

Conclusions—Direct gene delivery of HSP 70 in vivo reduces the severity of ischemic injury in the heart. (Circulation. 2001;103:877-881.)

Key Words: myocardial infarction ■ ischemia ■ proteins ■ genes ■ viruses

The stress response is commonly associated with a rapid overexpression of a family of heat-shock proteins. The major heat-shock proteins are a set of highly conserved proteins having molecular masses of 27, 70, 82, and 90 kDa. The most abundant and best-studied subset is the 70-kDa (HSP 70) protein family. This protein serves an important role by associating with nascently formed proteins that have not reached their permanent folding state and prevents their denaturation. The mechanism of action of HSP 70 and related proteins still remains largely a matter of speculation. There is now compelling genetic and biochemical evidence that these proteins belong to a family of ATP-dependent “unfoldases” implicated in protein assembly/dissociation of multimeric complexes, translocation, and import/export processes across membranes.

It is well established that preconditioning with brief episodes of ischemia, as well as other forms of stress, such as heat shock, result in increased production of HSP 70 in the heart. The amount of HSP 70 synthesis correlates with the extent of myocardial protection after ischemia/reperfusion injury. Experimental evidence supports the proposition that in vitro cardiac myocytes transfected with the gene encoding for HSP 70 are protected from ischemic injury. No work has been done, however, to show direct transfer of HSP 70 gene in vivo into the beating heart. In the present study, therefore, we sought to investigate the direct cause-and-effect relationship of HSP 70 overexpression in myocardial protection during prolonged ischemia/reperfusion injury in vivo in the rabbit heart. We used recombinant adenovirus encoding for the inducible form of human HSP 70 (Ad.HSP70) to transfer the gene into the cardiac muscle to show whether the increased expression of HSP 70 results in reduction of infarct size subsequent to ischemia/reperfusion injury.

Methods

Animals
Male New Zealand White rabbits (2.8 to 3.3 kg) were used for the studies. The rabbits were supplied by the Prince Rabbitry (Oakhill, WV) or the Blue and Gray Rabbitry (Unionville Lane, Va). The animals were allowed to readjust to the new housing environment for ≥1 week before the experiment. The care and use of the animals were conducted in accordance with the guidelines of the Committee on Animals of Virginia Commonwealth University.

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From the Division of Cardiology, Department of Medicine, Medical College of Virginia, Virginia Commonwealth University, Richmond (J.C.C., M.L.H., R.C.K.); the Department of Cardiology, Kanazawa Medical University, Ishikawa, Japan (S.O.); the Robert-Roessle-Klinik, Max-Delbrueck Center for Molecular Medicine, Berlin, Germany (O.W.); and Baylor College of Medicine, Houston, Tex (M.R.S.).

Correspondence to Rakesh C. Kukreja, PhD, Professor, Division of Cardiology, Medical College of Virginia, Virginia Commonwealth University, 1101 E Marshall St, Richmond, VA 23298. E-mail rakesh@hsc.vcu.edu

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mmol/L MgCl₂ and 10% glycerol 4 times at 4°C (1 hour each) with pJM1711 and pAVC3.HSP70 in 293 cells as previously described. 12 The virus titer was estimated by plaque assay on 293 cells. 14 The virus concentration was determined by measuring absorbency at 260 nm, 13 and the titer was estimated by plaque assay on 293 cells. The virus titer was 2×10¹⁰ plaque-forming units (pfu) per mL, and the particle-to-plaque ratio was ~80:1. The presence of replication-competent adenovirus in the plaque-purified Ad.HSP70 preparation was assessed by infecting A549 cells and excluded by the absence of lysis of the indicator cells 21 days after infection.

**Generation of the Adenoviral Construct**

The E1 region of the replication-defective adenoviral vector was replaced by an expression cassette containing the entire coding region for human heat-shock protein 70 (HSP70) driven by the human CMV-IE promoter in parallel to the transcriptional direction of the adenovirus E1 ORF and terminated by the simian virus 40 large tumor antigen gene. Ad.HSP70 was generated by cloning human HSP 70 from the plasmid pH2.3 (ATCC 57494, American Type Culture Collection) as a BamHI-ScaI fragment into the BamHI-PmeI sites of the adenoviral shuttle plasmid pAVC3. 10 In a subsequent step, Ad.HSP70 was rescued by homologous recombination of pJM1711 and pAVC3.HSP70 in 293 cells as previously described. 12 Ad.HSP70 was propagated in 293 cells, purified by 2 rounds of CsCl density centrifugation,12 dialyzed against 1500 mL of PBS with 1 mmol/L MgCl₂ and 10% glycerol 4 times at 4°C (1 hour each) with a Slide-A-Lyzer cassette (Pierce), and stored at

**Experiment Protocol**

The rabbits were randomly assigned into 1 of the following 3 groups: group 1, vehicle, hearts injected with saline alone; group 2, vector control, hearts injected with adenovirus encoding irrelevant gene, lacZ (Ad.lacZ); and group 3, Ad.HSP70, hearts injected with adenovirus Ad.HSP70. Separate hearts from each of groups 1 and 3 were used to evaluate the expression of HSP 70.

**In Vivo Gene Injection**

The animals were anesthetized with an intramuscular injection of ketamine HCl 35 mg/kg and xylazine 5 mg/kg. Further injections of ketamine/xylazine were given as needed throughout the surgical procedure. The animals were intubated orotracheally and ventilated on a positive-pressure ventilator. The tidal volume was set at ~15 mL, and the respiratory rate was adjusted to 30 to 40 cycles/min. Ventilator setting and PO₂ were adjusted as needed to maintain the blood gas parameters within the physiological range. The surgery was carried out under sterile conditions. A left thoracotomy was performed at the fourth intercostal space, and the heart was exposed to identify the coronary artery branch. A ligature was then placed around the left coronary artery, and the artery was occluded by snaring with a small tube through which the ligature had been passed. After 30 minutes of ischemia, the ligature was released and the heart reperfused for 3 hours.

**Myocardial Infarction Protocol**

Four days after the injection of saline or virus, the animals were reanesthetized and thoracotomy, artificially ventilated with room air. The thorax was reopened and the heart exposed to identify the coronary artery branch. A ligature was then placed around the left coronary artery, and the artery was occluded by snaring with a small tube through which the ligature had been passed. After 30 minutes of ischemia, the ligature was released and the heart reperfused for 3 hours.

**Measurement of Infarction**

At the end of the infarction protocol, the ligature around the coronary artery was retightened, and ~1 mL of 10% Evans blue dye was injected as a bolus into the jugular vein until the eyes turned blue. The animals were euthanized immediately, and the heart was removed and frozen. The heart was then cut from apex to base into 6 to 8 transverse slices of equal thickness. The area at risk was determined by negative staining with Evans blue. The slices were then incubated in 1% triphenyltetrazolium chloride solution in isotonic pH 7.4 phosphate buffer at 37°C for 20 minutes. The slices were subsequently fixed in 10% formalin solution for 6 hours.
Red-stained viable tissue was easily distinguished from the infarcted pale/unstained necrotic tissue. The areas of infarcted tissue, the risk zone, and the whole left ventricle (LV) were determined by computer morphometry with Bioquant imaging software. The area for each region was averaged from slices. Infarct size was expressed both as a percentage of the total LV and as a percentage of the ischemic risk area.

### Evaluation of Gene Transfer

Gene transfer in the LV and right ventricle (RV) of hearts treated with vehicle or Ad.HSP70 was evaluated by Western blot as described previously \(^{15}\) with a mouse monoclonal antibody cross-reacting to the HSP 70 or the constitutive form of HSP 70 (HSC 70) (Stressgen Biotechnologies Corp). The secondary antibody was horseradish peroxidase–conjugated rabbit anti-mouse IgG.

### Statistical Analysis

All measurements are expressed as group mean ± SEM. Changes in hemodynamics and infarct size variables were analyzed by a 1-way repeated-measures ANOVA to determine the effects of time, group, and time-by-group interaction. If the global tests showed major interactions, post hoc contrasts between different time points within the same group or between different groups were performed with a \(t\) test. Statistical differences with a value of \(P<0.05\) were considered significant.

### Results

#### Expression of HSP 70

In vivo intraventricular injection of Ad.HSP70 resulted in a robust expression of HSP 70 in the LV compared with the hearts injected with the vehicle (Figure 1). The noninjected area of the heart, ie, the RV, did not show HSP 70 expression in vehicle- or Ad.HSP70-treated hearts. Previous studies have shown that treatment of viral vectors has no effect on expression of HSP 70 in the myocytes.\(^{16,17}\) The expression of HSC 70 was not altered in LV and RV with the vehicle and Ad.HSP70 injections.

#### Myocardial Infarction

Ad.HSP70-injected hearts demonstrated significant reduction in infarct size (% risk area: 24.5 ± 2.8) compared with 41.9 ± 2.9 and 42.7 ± 2.5 in the vehicle- and Ad.lacZ-injected hearts, respectively \((P<0.01, \text{ Figure } 2\text{A})\). There were no significant differences in the infarct size between the vehicle- and Ad.lacZ-injected hearts (41.9 ± 2.9 versus 42.7 ± 2.5, \(P>0.05\)), suggesting that the reduced infarct size observed in Ad.HSP70-injected hearts was entirely due to the expression of HSP 70 but not HSC 70, which was uniformly expressed in the LV and RV. A similar pattern was observed when infarct size was expressed as percentage of LV (Figure 2B). The risk areas (% of LV) were not significantly different among the groups, ie, 50.1 ± 5.2, 47.7 ± 3.5, and 53.3 ± 2.9 in the vehicle-, Ad.lacZ-, and Ad.HSP70-treated groups, respectively \((P>0.05, \text{ Figure } 2\text{C})\).

#### Hemodynamics

Heart rate, mean arterial blood pressure, and rate-pressure product are shown in the Table. Except for the indicated differences, these parameters were comparable among the 3 groups at baseline, during occlusion, and during the reperfusion period. All groups had a similar decline in blood pressure after coronary occlusion. During reperfusion, the heart rate, mean arterial pressure, and rate-pressure product decreased gradually, sometimes significantly, as indicated in all the groups.

#### Discussion

Gene therapy is a rapidly expanding field with potential applications to every human organ system.\(^{18,19}\) Recently, adenoviruses have been used as efficient vectors for in vivo gene transfer into the myocardium.\(^{20}\) Using this delivery system, we set out to elucidate the direct cause-and-effect relationship of HSP 70 in inducing antinecrotic cardioprotection in the intact rabbit heart. Our results show that direct injection of Ad.HSP70 into the LV muscle caused robust expression of HSP 70 compared with the RV (the noninjected region). No significant increase in the expression of HSP 70 was observed in the vehicle-treated LV, suggesting that the increased expression in the Ad.HSP70-injected hearts was not due to injection-related stress. The expression of HSC 70 did

### Hemodynamic Data

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heart rate, bpm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>193 ± 11</td>
<td>217 ± 14</td>
<td>186 ± 8</td>
<td>185 ± 8</td>
<td>177 ± 9</td>
<td>168 ± 9</td>
<td>165 ± 12</td>
<td>159 ± 13</td>
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<tr>
<td>Ad.lacZ</td>
<td>197 ± 21</td>
<td>211 ± 19</td>
<td>192 ± 22</td>
<td>196 ± 21</td>
<td>191 ± 19</td>
<td>182 ± 17</td>
<td>170 ± 21</td>
<td>176 ± 17</td>
</tr>
<tr>
<td>Ad.HSP70</td>
<td>217 ± 14</td>
<td>221 ± 12</td>
<td>224 ± 13</td>
<td>213 ± 9</td>
<td>195 ± 8</td>
<td>185 ± 9</td>
<td>169 ± 9</td>
<td>162 ± 12</td>
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<td><strong>Mean arterial blood pressure, mm Hg</strong></td>
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<td></td>
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<tr>
<td>Vehicle</td>
<td>86 ± 5</td>
<td>80 ± 4</td>
<td>73 ± 8</td>
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<td>69 ± 2</td>
<td>66 ± 2</td>
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<tr>
<td>Ad.lacZ</td>
<td>92 ± 15</td>
<td>81 ± 13</td>
<td>83 ± 13</td>
<td>83 ± 14</td>
<td>79 ± 12</td>
<td>78 ± 12</td>
<td>79 ± 13</td>
<td>77 ± 12</td>
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<td>Ad.HSP70</td>
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<td>76 ± 2</td>
<td>77 ± 2</td>
<td>77 ± 1</td>
<td>72 ± 4</td>
<td>69 ± 3</td>
<td>74 ± 4</td>
<td>76 ± 7</td>
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<td><strong>Rate-pressure product, (10^{-3})</strong></td>
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<tr>
<td>Vehicle</td>
<td>18.9 ± 1.2</td>
<td>17.7 ± 0.9</td>
<td>17.0 ± 1.0</td>
<td>16.8 ± 1.0</td>
<td>15.0 ± 0.9</td>
<td>14.2 ± 1.1</td>
<td>13.0 ± 1.0</td>
<td>12.6 ± 1.0</td>
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<tr>
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<td>19.4 ± 0.8</td>
<td>18.5 ± 1.3</td>
<td>17.0 ± 1.3</td>
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<td>16.1 ± 1.6</td>
<td>15.4 ± 1.3</td>
<td>14.5 ± 1.3</td>
<td>14.9 ± 1.6</td>
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<tr>
<td>Ad.HSP70</td>
<td>22.0 ± 1.7</td>
<td>19.5 ± 1.0</td>
<td>19.9 ± 1.0</td>
<td>18.9 ± 0.8</td>
<td>17.1 ± 1.1</td>
<td>16.1 ± 1.0</td>
<td>14.4 ± 1.2</td>
<td>14.1 ± 1.6</td>
</tr>
</tbody>
</table>

Rate pressure product is heart rate × systolic pressure. Results are mean ± SEM. \(P<0.05\) vs baseline.
not change significantly in the LV and RV injected with vehicle, Ad.lacZ, or Ad.HSP70. The infarct area was significantly reduced in the ischemic hearts injected with Ad.HSP70. No significant difference in the infarct size was observed between the vehicle- (saline) and Ad.lacZ-injected hearts. Systemic hemodynamics, ie, heart rate, mean arterial pressure, and rate-pressure product during baseline (preisch- schemia), ischemia, and reperfusion, were not significantly different in the hearts treated with saline, Ad.lacZ, or Ad.HSP70. Taken together, our data show that direct delivery of HSP 70 with multiple injections of Ad.HSP70 into the LV muscle overexpressed the protein, which subsequently protected the heart against ischemia/reperfusion injury by reducing necrosis in the injected region of the heart.

A number of gene transfer approaches have been used, including direct injection of naked plasmid DNA, ex vivo genetically engineered transplanted cells, liposome-DNA complexes, and several recombinant and conjugated virus- es. Replication-defective adenoviruses have been exploited as gene delivery vehicles because of their ability to infect a wide variety of hosts and tissues. In addition, adenoviruses are highly efficient in infecting slowly replicating or nonreplicating cells, particularly myocytes. Direct injection of the adenovirus vectors into the heart muscle transduced genes in the transmural region of myocardium, which peaked during the first week but was completely extinguished at 30 days. Another study showed the expression of the gene to persist for as long as 55 days after injection, although the magnitude of expression was much lower. The cardiac myocytes were the target of the adenovirus-mediated gene transfer, as confirmed by histological examination. In the present study, we demonstrated increased expression of HSP 70 four days after injection of Ad.HSP70 into the LV, although long-term expression was not investigated. On the basis of the published studies with lacZ expression, it is possible that the duration of HSP 70 expression by our technique may have lasted for extended periods after in vivo injection. Further studies would be necessary to demonstrate the time course of gene expression with this technique and its correlation with ischemic tolerance in vivo.

Our direct gene transfer approach resulted in significant reduction in infarct size, suggesting that HSP 70 could be added into the number of gene products that can potentially be used clinically to reduce ischemic injury. A previous study demonstrated better functional recovery and less leakage of creatine phosphokinase after ischemia in the hearts trans- fected with HSP 70 gene than in the control or nontreated hearts. This study used a combination of intracoronary infusion of hemagglutinating virus of Japan liposome and heart transplantation to transfer the HSP 70 gene, followed by Langendorff perfusion to evaluate the effect of HSP 70 on myocardial protection. The intracoronary infusion technique caused global transfection of the HSP 70 gene in this study. In contrast, our approach is straightforward and involved direct injection of Ad.HSP70 into LV muscle in vivo, which resulted in localized expression of HSP 70.

Previous studies have shown distinct correlations between the expression of HSP 70 by pathophysiological stressors and cardiac resistance to ischemia. Transgenic mice overexpress-


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