Cell Transplantation for the Treatment of Acute Myocardial Infarction Using Vascular Endothelial Growth Factor–Expressing Skeletal Myoblasts

Ken Suzuki, MD, PhD; Bari Murtuza, MA, FRCS; Ryszard T. Smolenski, MD, PhD; Ivan A. Sammut, PhD; Noriko Suzuki, MD; Yasufumi Kaneda, MD, PhD; Magdi H. Yacoub, FRS

Background—Vascular endothelial growth factor (VEGF) is a promising reagent for inducing myocardial angiogenesis. Skeletal myoblast transplantation has been shown to improve cardiac function in chronic heart failure models by regenerating muscle. We hypothesized that transplantation of VEGF-expressing myoblasts could effectively treat acute myocardial infarction by providing VEGF-induced cardioprotection through vasodilatation in the early phase, followed by angiogenesis effects in salvaging ischemic host myocardium combined with the functional benefits of newly formed, skeletal myoblast-derived muscle in the later phase.

Methods and Results—Primary rat skeletal myoblasts were transfected with the human VEGF<sub>165</sub> gene using hemagglutinating virus of Japan-liposome with >95% transfection efficiency. Four million of these myoblasts (VEGF group), control-transfected myoblasts (control group), or medium only (medium group) was injected into syngeneic rat hearts 1 hour after left coronary artery occlusion. Myocardial VEGF-expression increased for 2 weeks in the VEGF group, resulting in enhanced angiogenesis without the formation of tumors. Grafted myoblasts had differentiated into multinucleated myotubes within host myocardium. Infarct size (33.3±1.4%, 38.1±1.4%, and 43.7±1.6% for VEGF, control, and medium groups, respectively; P=0.0005) was significantly reduced with VEGF treatment, and cardiac function improved in the VEGF group (maximum dP/dt: 4072.0±93.6, 3772.5±101.1, and 3482.5±90.6 mm Hg/s in the 3 groups, respectively; P=0.0011; minimum dP/dt: -504.2±68.5, -2311.3±57.0, and -2124.0±57.9 mm Hg/s, respectively; P=0.0008).

Conclusions—This combined strategy of cell transplantation with gene therapy could be of importance for the treatment of acute myocardial infarction. (Circulation. 2001;104[suppl I]:I-207-I-212.)

Key Words: cells n transplantation n gene therapy n angiogenesis n myocardial infarction

Vascular endothelial growth factor (VEGF) is a promising therapeutic reagent for treating myocardial infarction by inducing angiogenesis. However, sustained, localized high levels of VEGF expression in myocardium are needed for its clinical use; such levels may be achieved by genetic manipulation. It has been reported that direct intramyocardial gene transfer results in localized enhancement of VEGF levels and successful angiogenesis in animal models of myocardial infarction. Further, preliminary data from recent human trials of angiogenesis gene therapy using naked plasmid DNA or an adenoviral vector coding for VEGF have shown favorable results. Cell-mediated gene transfer may also be useful for sustained, local protein delivery. In addition to its angiogenesis effect, VEGF reportedly provides myocardial protection against ischemic injury via vasodilatation mediated by an increase in nitric oxide.

It has been shown that skeletal myoblasts (satellite cells) survive within myocardium, thus regenerating functional muscle and improving cardiac function when transplanted in experimental heart failure models. Thus, skeletal myoblast transplantation is promising for treating chronic end-stage heart failure; however, the efficiency of this strategy when applied in the acute phase of myocardial infarction remains unknown. In the present study, we investigated the feasibility and efficiency of transplanting skeletal myoblasts that are transiently expressing VEGF to treat acute myocardial infarction. We hypothesized that this strategy could provide cardioprotective effects for ischemic host myocardium by VEGF-induced vasodilatation in the early phase of infarction, followed by angiogenesis effects in salvaging host myocardium in combination with functional benefits of newly formed, skeletal myoblast–derived muscle by cellular cardiomyoplasty in the late phase.

Methods

Isolation of Primary Skeletal Myoblasts

All studies were performed with the approval of the institutional ethics committee. The investigation conformed to the Principles of

From the Department of Cardiothoracic Surgery, Imperial College School of Medicine, Heart Science Centre, Harefield Hospital, Middlesex, UK, and the Division of Gene Therapy Science, Osaka University, Osaka, Japan (Y.K.). Correspondence to Professor Sir Magdi H. Yacoub, Department of Cardiothoracic Surgery, Harefield Hospital, Harefield, Middlesex, UB9 6H, UK. E-mail k.suzuki@ic.ac.uk © 2001 American Heart Association, Inc. Circulation is available at http://www.circulationaha.org
A

B

C

D

Figure 1. Primary skeletal myoblasts were isolated from single fibers of rat skeletal muscle. Skeletal myoblasts dissociated from the fiber 12 to 24 hours after plating (A and B; reverse-phase contrast microscope observation). All cells demonstrated α-sarcomeric actin expression (FITC; green) by immunocyto-tostaining. Nuclei were counterstained blue with 4,6-diamino-2-phenylindole (C). When cultured in differentiation medium, the myoblasts fused and differentiated into multinucleated myotubes (D). Scale bar, 250 μm for A and 50 μm for B, C, and D.

Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Primary skeletal myoblasts were isolated using the single-fiber culture system. The extensor digitorum longus was removed from a male Lewis rat (80 g) and was digested with 0.2% type I collagenase (Sigma). Single, intact muscle fibers liberated by trituration were placed into each well of a 24-well culture plate. Putative skeletal myoblasts (satellite cells) dissociated from the fiber, appeared 12 to 24 hours after plating, and proliferated (Figures 1A and 1B). The fiber was then removed and the myoblasts were fed with proliferation medium (20% fetal calf serum, 10% horse serum, and 0.5% chicken embryo extract in Dulbecco’s Modified Eagle Medium [DMEM]). The culture was passaged before reaching 75% confluence to maintain the undifferentiated state. To confirm the purity of the culture, cells were seeded into 4-well chamber slides (Labtek). Incubating with 5% horse serum, the samples were incubated in a 1:5 dilution of anti-α-sarcomeric actin monoclonal antibody (Dako) at 4°C overnight, followed by 1.5 hours of incubation in a 1:200 dilution of FITC-conjugated secondary antibody (Dako). Nuclei were counterstained with 0.1 mg/mL propidium iodine.

Gene Construction and Gene Transfection

Full-length cDNA for human VEGFα was generously donated by Dr Y. Yonemitsu (Kyushu University, Japan), which was directionally cloned into the EcoRI site of pcDNA3.1′ vector (Invitrogen Corporation). Hemagglutinating Virus of Japan (HJV)-cationic-liposome was prepared as described previously. Briefly, 200 μg DNA was mixed with a lipid film composed of 10 mg of a lipid mixture (phosphatidylserine, dimethylaminoethane-carbamoyl cholesterol, and cholesterol) to generate a liposome-DNA complex suspension. The suspension was then incubated with 30 000 hemagglutinating units of inactivated HVJ by ultraviolet light, resulting in 1 mL of HVJ-liposome. Skeletal myoblasts at 50% confluence in a 175 cm² flask were incubated with 450 μL of the HVJ-liposome containing the human VEGFα gene in pcDNA3.1′ in 20 mL of proliferation medium for 2 hours at 37°C. Control cells were transfected with pcDNA3.1′ without the VEGF gene using the same protocol.

In Vitro VEGF Expression

At day 3 after gene transfection, the culture medium was collected from VEGF- or control-transfected myoblasts (n=6 in each group). VEGF levels in the medium were quantified by an ELISA kit for human VEGF (Chemicon International) by following the company’s instructions using a microplate reader (Labosystems). After collecting medium, myoblasts were harvested by scraping confluent, 175-cm² plates in 2 mL of 1% sodium dodecyl sulfate (SDS) containing 10 μg/mL leupeptin, 1 mM phenylmethyl sulfonyl fluoride, and 5 μg/mL aprotinin. After homogenization and centrifugation at 35 000g for 15 minutes, the protein concentration of the supernatant was measured using the Bradford protein assay method (Bio-Rad). A total of 25 μg of protein from each sample was loaded onto a SDS 10% polyacrylamide gel for electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked and incubated with a 1:1000 dilution of anti-human VEGF monoclonal antibody (Santa Cruz Biotechnology) at 4°C overnight, followed by 1-hour incubation with a 1:1000 dilution of horseradish peroxidase–conjugated secondary antibody (Sigma). The blot was visualized with an enhanced chemiluminescent detection system (Amersham). The films were scanned using a Molecular Dynamics 300A laser densitometer to determine VEGF levels using Quantity One software (PDI). Data are shown as the relative density of bands versus day 2 samples from medium-injected hearts.

For immunocytochemical analysis, myoblasts were seeded into 2-well chamber slides (Nunc) at day 2 after gene transfection. The next day, samples were fixed with ice-cold ethanol. After incubation in 0.1% Triton X-100 and 1% bovine serum albumin, the samples were incubated in a 1:50 dilution of anti-human VEGF antibody (Santa Cruz Biotechnology) at 4°C overnight, followed by incubation in a 1:20 dilution of FITC-conjugated secondary antibody (Dako). Nuclei were counterstained with 0.1 mg/mL propidium iodide.

Myocardial Infarction Followed by Cell Transplantation

Male Lewis rats (250 g) were anesthetized with sodium pentobarbital (50 mg/kg IP) and mechanically ventilated. After the heart was exposed through a lateral thoracotomy, a 6-0 polypropylene thread was passed around the left coronary artery, ~3 mm distal to its origin, and the artery was occluded. At day 3 after gene transfection, myoblasts were harvested using trypsin and resuspended in serum-free DMEM just before grafting to the heart. At 1 hour after left coronary artery occlusion, the VEGF- or control-transfected skeletal myoblasts (VEGF and control groups, respectively) or serum-free DMEM only (medium group) was injected into the border zone surrounding the infarct (4 injections of 1×10⁶ cells in 100 μL) with a 27G needle. The surgical wounds were repaired, and the rats were returned to their cages to recover. Aseptic surgical techniques were used throughout.

Myocardial VEGF Level after Cell Transplantation

At 2, 4, 7, 14, and 28 days after cell transplantation, 5 hearts from each group were collected to assess the VEGF level of the heart. The isolated left ventricle was cut into small pieces, immediately frozen in liquid nitrogen, and homogenized with a Polytron homogenizer at 10 000 rpm for 30 s in ice-cold PBS. After 30 s of sonication, the homogenate was centrifuged at 35 000g for 15 minutes. The supernatant (100 μg of protein) was applied to Western blotting for VEGF, and the band density on the film was determined by densitometry using analysis-software as described above. To enable comparison, the band density at each time point was determined relative to the value obtained at day 2 in the medium-injected hearts.
Functional Assessment
At 28 days after cell transplantation, rats from 3 groups (n=8 in each group) were anticoagulated by an intravenous injection of heparin. The hearts were quickly excised and perfused at a pressure of 1 m H2O with modified Krebs-Henseleit buffer (120.0 mmol/L NaCl, 4.5 mmol/L KCl, 20.0 mmol/L NaHCO3, 1.2 mmol/L KH2PO4, 1.2 mmol/L MgCl2, 1.25 mmol/L CaCl2, and 10.0 mmol/L glucose; gassed with 95% O2 plus 5% CO2 at 37°C) using a Langendorff apparatus.9 After 20 minutes of stabilization, heart rate, ventricular function, and coronary flow were measured after left ventricular end-diastolic pressure was stabilized at 10 mm Hg using a thin-walled balloon. Coronary flow was recorded with an electromagnetic flowmeter (Scalar).

Infarct Size, Grafted Myoblasts, and Angiogenesis
After perfusion, the left ventricle was sectioned into 5 segments parallel to the apex-base axis and frozen in an embedding medium. A 10-μm section was cut from each segment, fixed in 3.7% formaldehyde, and stained with Masson trichrome. Sections from all slices were projected onto a screen for computer-assisted planimetry. The ratio of scar length to left ventricular circumferences of the endocardium and epicardium was expressed as a percentage to define infarct size.16

The frozen left ventricular samples were cut into 6-μm sections and fixed with –20°C methanol. The sections were incubated with 0.6% H2O2, and immersed in 0.1% Triton X-100. After blocking with 1% bovine serum albumin, the sections were incubated with a 1:40 dilution of anti-skeletal myosin heavy chain antibody (Zymed Laboratories). This was followed by incubation with a 1:100 dilution of biotinylated secondary antibody (Dako). The sections were colored with a Dako streptABCComplex/horseradish peroxidase Duet kit and counterstained with hematoxylin and eosin. The number of capillary vessels was counted in the peri-infarct area using a light microscope at a magnification of ×400.17 Five high-power fields in each section were randomly selected, and the number of capillaries in each field was averaged and expressed as the number of capillary vessels per high-power field (0.2 mm²).

Statistical Analysis
All values are expressed as mean±SEM. The differences in the data between 2 groups were determined with a Student’s t test. Mortality (survival rate) was analyzed using the Kaplan-Meier method followed by the log rank test. Statistical comparison of the data regarding myocardial VEGF expression was performed using 2-way repeated-measures ANOVA followed by Bonferroni/Dunn post hoc testing. Analysis of infarct size, cardiac function, and capillary density was performed with one-way ANOVA followed by Bonferroni/Dunn post-hoc testing. P<0.05 was considered significant.

Results
In Vitro VEGF-Gene Transfection of Primary Skeletal Myoblasts
During culture in proliferation medium, the myoblasts isolated from the extensor digitorum longus of Lewis rats grew to confluence and, within a week of switching to low-serum medium, they fused and differentiated into multinucleated myotubes (Figure 1D). Immunocytostaining for α-sarcomeric actin demonstrated that all the cells were positively stained, suggesting the high purity of the culture (Figure 1C). On day 3 after gene transfection with human VEGF165 using HVJ-liposome, >95% of transfected myoblasts were positively stained for VEGF (Figure 2C). The level of secreted VEGF in the culture medium of VEGF-transfected myoblasts was significantly higher compared with control-transfected myoblasts, as measured by ELISA (2776.0±200.7 versus 122.4±10.9 pg/mL, P<0.0001; Figure 2A). Similarly, a higher (20.3±4.5-fold by densitometric quantification) VEGF level in the cell lysate of VEGF-transfected myoblasts was confirmed by Western blotting (Figure 2B).

Mortality and VEGF Expression in Myocardium After Cell Transplantation
At 1 hour after left coronary artery occlusion, the border zones of infarcts were injected with VEGF- or control-vector mediated by HVJ-liposome. The mortality over 28 days after cell transplantation was significantly reduced in the VEGF group (5 deaths in 48 rats, 10.4%; P=0.0087 versus medium group; P=0.0120 versus control group) compared with the medium group (14 deaths in 55 rats, 25.5%) and control group (11 deaths in 52 rats, 21.2%; P=0.0812 versus medium group). The majority of the deaths were observed within 24 hours after treatment. Western blot analysis demonstrated that myocardial VEGF levels in the VEGF group increased significantly (P=0.0008) between days 2 and 14 after transplantation, peaking at day 4 (9-fold over the day 2 sample for medium group), compared with the other groups (P=0.0012 versus medium group; P=0.0081 versus control group; Figure 3). The VEGF level of the control group tended to be higher (P=0.0521) than that of the medium group over the period studied.

Infarct Size and Cardiac Function After Cell Transplantation
At day 28 after cell transplantation, infarct size was significantly reduced in the VEGF group compared with other groups, and the control group demonstrated a reduced infarct size in comparison with the medium group (Table). These findings correlated inversely with coronary flow measured...
using Langendorff perfusion. Both maximum and minimum dP/dt at 10 mm Hg of left ventricular end-diastolic pressure were best in the VEGF group, indicating that both systolic and diastolic functions were best preserved in the VEGF group after myocardial infarction. Further, these indicators were better in the control group than in the medium group, suggesting that skeletal myoblast transplantation alone could preserve cardiac function after myocardial infarction.

**Grafted Myoblasts and Angiogenesis**

Groups of cells that were positively stained for skeletal myosin heavy chain were observed locally in the peri-infarct area at 28 days after the transplantation of control- and VEGF-transfected myoblasts (Figures 4B and 4C). Because this antibody reacts with skeletal myosin heavy chain but not with cardiac or smooth muscle myosin, these positively stained cells were presumed to be of skeletal myoblast origin. It was demonstrated that surviving myoblasts had differentiated into multinucleated myotubes that had aligned with the cardiac fiber axis within the native myocardium (Figure 4D). Positively stained myotubes were observed at almost all injection sites studied in the VEGF and control groups. Although not accurately quantified in this study, examination of random high-power fields on light microscopy indicated that the number of positively stained myotubes was larger in the VEGF group than in the control group. Angiogenesis, as defined by capillary number, was found in the area surrounding grafted skeletal myoblasts of the control group (5.0 ± 0.4 capillaries per high-power field; \( P = 0.0046 \) versus medium group; Figure 5A), but obvious angiogenesis was not found in the Medium group (2.5 ± 0.2 capillaries per high-power field). Further, enhanced angiogenesis with a larger number of mature capillaries was observed in the VEGF group (8.7 ± 1.4 capillaries per high-power field; \( P = 0.0001 \) versus medium group, \( P = 0.0055 \) versus control group; Figures 5B and 5C). There was no tumor formation in any heart studied.

**Discussion**

We demonstrated that the transplantation of control-transfected skeletal myoblasts can reduce infarct size and improve cardiac function after left coronary artery occlusion compared with medium injection. Moreover, it has been shown that the grafting of skeletal myoblasts expressing VEGF provides further advanced benefits in reducing infarct size and preserving cardiac function; these effects correlated with the enhanced angiogenesis observed in comparison with control-myoblast transplanted hearts. These data suggest that

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**Infarct Size and Cardiac Function After Cell Transplantation**

<table>
<thead>
<tr>
<th>Group</th>
<th>Infarct Size, %</th>
<th>Heart Rate, bpm</th>
<th>LVDP, mm Hg</th>
<th>Maximum dP/dt, mm Hg/s</th>
<th>Minimum dP/dt, mm Hg/s</th>
<th>Balloon Size, ( \mu L )</th>
<th>Coronary Flow, mL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>43.7 ± 1.6</td>
<td>252.4 ± 12.5</td>
<td>114.3 ± 3.4</td>
<td>3482.5 ± 90.6</td>
<td>-2124 ± 57.9</td>
<td>172.9 ± 5.8</td>
<td>10.1 ± 0.3</td>
</tr>
<tr>
<td>Control</td>
<td>38.1 ± 1.4*</td>
<td>242.8 ± 13.0</td>
<td>129.8 ± 3.8*</td>
<td>3772.5 ± 101.1*</td>
<td>-2311.3 ± 57.0*</td>
<td>198.6 ± 6.1*</td>
<td>11.2 ± 0.4*</td>
</tr>
<tr>
<td>VEGF</td>
<td>33.3 ± 1.4†</td>
<td>257.8 ± 11.8</td>
<td>142.2 ± 4.1†</td>
<td>4072.0 ± 93.6†</td>
<td>-2504.2 ± 68.5†</td>
<td>221.5 ± 6.7†</td>
<td>12.3 ± 0.4†</td>
</tr>
</tbody>
</table>

\( P = 0.0005 \) 0.0032 0.0011 0.0008 0.0023 0.0017

Data were measured at day 28 after cell transplantation. Functional data were measured at 10 mm Hg of left ventricular end-diastolic pressure. Data are presented as mean ± SEM. LVDP indicates left ventricular developed pressure. \( n = 8 \) in each group.

\* \( P < 0.05 \) vs medium group; † \( P < 0.05 \) vs both medium and control groups.
transplantation of VEGF-expressing myoblasts could be of significant value in treating acute myocardial infarction.

We speculate that this strategy could, in the early phase of infarction, provide cardioprotective effects through VEGF-induced vasodilatation as mediated by an increase in nitric oxide production, independently of angiogenesis.7,8 To clarify if locally expressed VEGF effectively contributes to myocardial protection, further studies, such as an assessment of cardiac function and infarct size in the early phase after the insult, are needed. In the late phase, in turn, the angiogenesis effect in salvaging ischemic host myocardium, combined with cellular cardiomyoplasty effects in regenerating muscle could play an additional role in improving function. Further, the cellular cardiomyoplasty effect could be reinforced by improved graft survival resulting from an improved blood supply to grafted myoblasts through both vasodilatation and enhanced angiogenesis. This would be particularly beneficial in the early stage after cell transplantation, when grafted cells are subjected to various pathological processes caused by environmental stress, such as ischemic and mechanical injury.18 Such stress is known to result in both the necrosis and apoptosis of grafted myoblasts.10,19 Although it has been reported that skeletal myoblast transplantation improves cardiac function in heart failure models,10,11 we think that the cellular cardiomyoplasty effect could be reinforced by direct intramyocardial gene overexpression mediated by direct intramyocardial gene transfer might be sufficient to induce collateral vessels in ischemic myocardium but not enough to produce tumors.3–5

The present strategy could achieve an adequate magnitude and duration (≈2 weeks) of VEGF expression in the ischemic myocardium as a result of implanting VEGF-expressing myoblasts by transient gene transfection mediated by HVJ-liposome, which is more likely to induce functional collateral vessels without angiogenesis for up to 28 days after treatment.

To exclude the possibility of tumorigenesis using the current strategy, further study with longer-term incubation might be necessary. In addition, by using myoblasts as a platform for VEGF delivery, one would anticipate a continued functional benefit from newly formed skeletal myotubes. However, further investigations are needed to clarify exactly how long and how much VEGF expression is optimal to treat myocardial infarction. We have also demonstrated that angiogenesis is induced in infarcted hearts after control-myoblast transplantation and that this is associated with increased myocardial VEGF levels. This finding has been observed in previous reports using direct intramyocardial injection of skeletal myoblasts.21 In addition, other studies have shown that a nonspecific reaction caused by mechanical injury results in angiogenesis induction in the heart.22 One might speculate that mechanical injury by cell injection and the presence of foreign cells in myocardium might stimulate endogenous expression of some growth factors, including VEGF.

The optimal graft cell number is that required to achieve the best attenuation of adverse remodeling or improvement in cardiac function after skeletal myoblast transplantation. In this study, we showed that the injection of 4 million skeletal myoblasts results in reduced infarct size and improved function after acute myocardial infarction. Taylor et al demonstrated that a direct intramuscular injection of 10^7 skeletal myoblasts improved the function of cryoinjured rabbit hearts, whereas Scorsin and coworkers showed that 5 million myoblasts improve function in rats after infarction. However, the overall functional result may be affected by other factors, such as graft survival, differentiation capacity, ability in forming gap junctions with host myocardium, and the condition of host myocardium. In this study, we did not accurately measure the graft survival rate; however, it is likely that VEGF expression would be useful in improving graft survival according to histological observations. Further study of the quantitative assessment of survival by measuring β-galactosidase (β-gal) activity (transplantation of β-gal–expressing skeletal myoblasts) or detecting the Y chromosome (transplantation of male grafts into female hearts) would be needed to clarify this issue. We recently demonstrated that prior heat shock treatment of grafted myoblasts enhances self-preservation systems against environmental stress and improves their survival after engraftment to the heart using β-gal–expressing skeletal myoblasts.19 To compare the effect on graft survival between heat shock treatment, VEGF expression, and the combination of both strategies would be interesting as future work.

Gap junction formation between grafted myoblasts and host cardiomyocytes is an important but controversial issue concerning skeletal myoblast transplantation to the heart. We
did not investigate this point in the present study because of technical difficulty in assessing functional gap junctions in vivo; however, we have reported that gap junctions, as identified by connexin 43 expression, are formed between grafted skeletal myoblasts and host cardiomyocytes after the intracoronary infusion of skeletal myoblast.7 Future study is needed to clarify such gap junction formation after direct myocardial injection.

The timing and site of cell grafting is an interesting issue of considerable debate. We injected cells 1 hour after left coronary artery ligation, simulating the treatment of acute coronary occlusion. Early injection of VEGF-overexpressing cells could be useful in salvaging more myocardium due to the vasodilatation effect of secreted VEGF. However, it is likely that grafted cells suffer greater adverse conditions if transplanted at this time point, in comparison with delayed injection after the myocardium has been stabilized. In addition, in the present study, we grafted myoblasts in the border area of infarcts because salvage of stunned or hibernating myocardium in this area could be of the most significance. Investigating the effects of varying injection times (in the acute, subacute, and chronic phase) or injection sites (into the center or border of infarct) would provide new and interesting data on cell transplantation to the heart.

The use of the HVJ-liposome has been shown to result in a transient and high-efficiency transfection, with little cell toxicity.14–15 Further, repetitive transfection using this vector is successful in vivo, suggesting a low immunogenicity of HVJ-liposome.23 In addition, our method requires the use of only inactivated HVJ in vitro, minimizing the safety concerns related to other viral gene therapy protocols such as viral replication, aberrant expression of viral genes, and alterations of host genomic structure. These features would be of significance in clinical applications of this strategy.

In summary, we have demonstrated that the transplantation of VEGF-expressing skeletal myoblasts by HVJ-liposome–mediated gene transfection results in transient, high-level VEGF expression within rat myocardium suffering from acute infarction. This expression leads to successful angiogenesis, which is associated with reduction in infarct size and an improvement in cardiac function, without tumor formation. This clinically relevant, combined strategy could be of importance for treating patients with acute myocardial infarction.

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References
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