Development of a Novel Method for Cell Transplantation Through the Coronary Artery

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**Background**—Cell transplantation is a promising strategy to treat end-stage heart failure. At present, a popular method to deliver cells into the heart is direct intramuscular injection. This method, however, may not be efficient in spreading cells globally into the myocardium. We have developed a novel method for cell transplantation using intracoronary infusion.

**Methods and Results**—An L6 rat skeletal muscle cell line expressing β-galactosidase (β-gal) was generated by gene transfection and clonal selection. These cells (10⁶ in 1 mL medium) were infused into explanted rat hearts through the coronary artery, followed by heterotopic heart transplantation into the abdomen of recipients. Control hearts were infused with cell-free medium. According to β-gal activity measurements, 5 × 10⁵ grafted cells per heart existed on day 3, increasing to 5 × 10⁶ on day 28 in the cell-transplanted hearts. At day 28, discrete loci positively stained for β-gal were observed throughout the cardiac layers of both left and right coronary territories. Some of them differentiated into β-gal–positive multinucleated myotubes that aligned with the cardiac fiber axis and integrated into the native myocardium, whereas others formed colonies consisting of undifferentiated myoblasts. Connexin 43, a cardiac gap junction protein, was expressed between grafted cells and native cardiomyocytes. No reduction in cardiac function was observed in a Langendorff perfusion system.

**Conclusions**—We have developed a unique method for efficient cell transplantation based on intracoronary infusion. This method, potentially applicable in the clinical setting during cardiac surgery, could be useful to globally supply cells to the heart. *(Circulation. 2000;102[suppl III]:III-359-III-364.)*

**Key Words:** cells | transplantation | heart failure

Although death from heart disease has shown a steady decline for the past decade, the prevalence of congestive heart failure continues to increase. At present, the effect of drug treatment is limited, and thus heart transplantation is the only established way to efficiently treat patients with end-stage heart failure. There are some serious disadvantages, however, such as complications from immunosuppressive agents and high cost.1,2

Cell transplantation is a promising strategy to treat end-stage heart failure. The ability to augment the number of cardiomyocytes could be of therapeutic value if the new myocytes functionally integrated with preexisting myocardium. Several types of cells have been used as a graft in cell transplantation models, demonstrating successful long-term survival in the mammalian myocardium.3–13 Recently, it has also been reported that cell transplantation could improve cardiac function of damaged heart.4–7 The popular method for cell delivery into the heart described in the reports is direct intramuscular injection, in which 0.5 to 10⁶ cells can be infused into the myocardium through a small thoracotomy.4–9,12,13 This method enables one to transplant cells selectively into either intact or infarcted parts of the myocardium. One cannot, however, avoid mechanical injury and induction of an inflammatory response, resulting in myocardial damage to some extent. In addition, this grafting system may not be advantageous in spreading cells globally into the myocardium. Cells infused by this method usually produce localized islet-like formations.4–9,12,13 resulting in limitation of the cell-to-cell interaction between grafted cells and native cardiomyocytes. We speculate that this may limit integration of the grafted cells into native myocardium, restricting the efficiency of cell transplantation. We have, in the present study, developed a unique system for global dissemination of cells into the myocardium by using intracoronary infusion.

**Methods**

**Animal Care**

All studies were performed with the approval of the institutional ethics committee for animal research. The investigation conforms to the Principles of 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 (NIH Publication No. 85-23, revised 1996).
Cell Culture and Generation of \( \beta \)-Galactosidase–Expressing L6 Skeletal Myoblasts

The L6 rat skeletal muscle cell line (American Type Culture Collection) was maintained with Dulbecco’s modified Eagle’s medium (DMEM, Sigma) supplemented with 2 mmol/L glutamine, 50 IU/mL penicillin, 50 \( \mu \)g/mL streptomycin, and 10% FCS. LacZ reporter gene was transfected into L6 myoblasts with MFG\( \text{Gn} \)las\( \text{LZ} \) retrovirus-mediated gene transfection as described before.\(^{14} \) Approximately 50% of cells expressed nuclear \( \beta \)-galactosidase (\( \beta \)-gal). Clonal cells expressing \( \beta \)-gal were selected by following 2 rounds of limiting dilution at a concentration of 0.2 cell/well. Cells were incubated at 37°C in a humidified chamber equilibrated with 5% \( \text{CO}_2 \) in air. The culture was passaged before reaching 80% confluence to maintain the undifferentiated state and used before the 12th passage.

Evaluation of \( \beta \)-Gal Expression

\( \beta \)-gal expression in the cells was confirmed with \( \beta \)-gal staining in vitro and Western blotting. For \( \beta \)-gal staining, cells were incubated on 8-well chamber slides (Nunc). After fixation in 2% formaldehyde and 0.2% glutaraldehyde, cells were washed and incubated with 1 mg/mL 5-bromo-4-chloro-3-indoyl-\( \beta \)-d-galactopyranoside, 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, and 2 mmol/L MgCl\(_2\); for 24 hours at 37°C.\(^{3,14,15} \)

For Western blotting, cells were harvested by scraping confluent 60-mm plates in 500 \( \mu \)L of 1% SDS containing 1 mmol/L phenylmethylsulfonyl fluoride, 5 \( \mu \)g/mL leupeptin, and 5 \( \mu \)g/mL aprotonin and then homogenated. After 30-second sonication, 25 \( \mu \)g of protein was loaded onto an SDS 10% polyacrylamide gel. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane. After blocking nonspecific binding sites, the membrane was immunoreacted with a 1:1000 dilution of anti-\( \beta \)-gal mouse monoclonal antibody (Sigma) for 8 hours at 4°C. The blots were then incubated with a 1:1000 dilution of horseradish peroxidase–conjugated goat anti-mouse IgG antibody (Dako) for 1 hour. Blots were visualized with the use of a chemiluminescence detection system (Amersham).

Intracoronary Infusion of Skeletal Myoblasts

The myoblasts expressing \( \beta \)-gal were harvested with the use of trypsin, resuspended in serum-free DMEM at a concentration of 2\(^*\)10\(^6\) cell/mL, and stored at 4°C until infusion to the heart (<30 minutes). The hearts of Sprague-Dawley rats (250 g) were arrested with cold crystalloid cardioplegic solution and removed under anesthesia with sodium pentobarbital (50 mg/kg IP) and anticoagulation with heparin (200 USP units IV). Just before infusion, the cells were filtered through a 20-\( \mu \)m membrane filter (Millipore) and adjusted to a concentration of 1\(^*\)10\(^6\) cell/mL in serum-free DMEM.

The hearts were infused with 1\(^*\)10\(^6\) cells through the coronary artery, with the venae cavae, pulmonary arteries, and veins ligated (group CTx). For the control group, the same volume of cell-free DMEM was infused. After incubation under increased intracoronary pressure on ice for 10 minutes, the pulmonary artery was incised and the coronary circulation flushed with 10 mL of cold PBS to remove excess myoblasts. The hearts were then transplanted into the abdomens of recipient rats (350 g) of the same strain.\(^{16,17} \) These hearts were collected on days 0 (10 minutes after cell infusion without heart transplantation), 3, 7, 14, and 28 after cell transplantation.

Functional Assessment of the Heart in a Langendorff Perfusion System

At the selected time, recipient rats were anticoagulated by intravenous injection of heparin under terminal anesthesia. The transplanted hearts were quickly excised from the abdomen and perfused with modified Krebs-Henseleit buffer (120.0 mmol/L NaCl, 4.5 mmol/L KCl, 20.0 mmol/L NaHCO\(_3\), 1.2 mmol/L KH\(_2\)PO\(_4\), 1.2 mmol/L MgCl\(_2\), 1.25 mmol/L CaCl\(_2\), and 10.0 mmol/L glucose; gassed with 95% O\(_2\)–5% CO\(_2\) at 37°C) at a 1 mL H\(_2\)O pressure with a Langendorff apparatus. A thin-wall balloon was inserted into the left ventricle to monitor left ventricular pressure. After 20 minutes of stabilization, functional parameters were measured with left ventricular diastolic pressure stabilized at 10 mm Hg.\(^{16} \)

Assay for \( \beta \)-Gal Activity

Assay for \( \beta \)-gal activity was performed to evaluate the number of transplanted cells existing in the heart.\(^{14,15} \) After Langendorff perfusion, the hearts (\( n = 7 \) at each point for each group) were immediately frozen in liquid nitrogen and homogenized in 0.25 mol/L Tris-HCl (pH 7.8). The homogenates were centrifuged at 3500g for 5 minutes and then 12 000g for a further 5 minutes. Thirty microliters of the supernatant was mixed with 66 \( \mu \)L of 4 mg/mL ONPG (O-nitrophenyl-\( \beta \)-d-galactopyranoside; Sigma) dissolved in 0.1 mol/L sodium phosphate (pH 7.5), 3 \( \mu \)L of 4.5 mol/L \( \beta \)-mercaptoethanol dissolved in 0.1 mol/L MgCl\(_2\), and 201 \( \mu \)L of 0.1 mol/L sodium phosphate. The mixture was incubated 37°C for 30 minutes and the reaction was stopped by adding 500 \( \mu \)L of 1 mol/L Na\(_2\)CO\(_3\). OD was read on a spectrophotometer at a wavelength of 420 nm. The value was divided by protein concentration measured with the Bradford assay.\(^{15} \)

Standard Scale of \( \beta \)-Gal Activity

A standard scale was produced to evaluate the number of myoblasts existing in the heart from the data of \( \beta \)-gal activity. Hearts were removed from nontreated rats under terminal anesthesia, perfused with a Langendorff apparatus to wash out blood, and mixed with known numbers (1\(^*\)10\(^3\), 1\(^*\)10\(^4\), 1\(^*\)10\(^5\), 5\(^*\)10\(^4\), or \( 1 \times 10^5 \)) of \( \beta \)-gal–expressing L6 myoblasts. The mixtures (5 samples in each group) were then homogenated to measure \( \beta \)-gal activity of the samples as described above.

In Situ Staining for \( \beta \)-Gal and Connexin 43

The remaining hearts from both groups (\( n = 5 \) at each point for each group) were frozen in an embedding medium with liquid nitrogen for in situ \( \beta \)-gal staining. The embedded samples were cut into 10-\( \mu \)m sections, fixed, and stained as above. For immunohistochemical study of connexin 43 (Cx43), after \( \beta \)-gal staining, the sections were blocked with 5% FCS and incubated for 1 hour in a 1:50 dilution of anti-Cx43 mouse monoclonal antibody (Chemicon) at room temperature. After washing, these were followed by 1 hour incubation in a 1:100 dilution of biotinylated goat anti-mouse IgG antibody (Dako) at room temperature. Coloring was performed with a kit (StreptAB-Complex/HRP; Dako) followed by counterstaining with 1% neutral red for 10 minutes.

Statistical Analysis

All values are expressed as mean±SEM. Statistical comparison of the data for \( \beta \)-gal activity was performed with ANOVA for repeated measures followed by Bonferroni’s test to individual significant difference. The differences in the data for cardiac function were determined with the Student’s \( t \) test. A value of \( P < 0.05 \) was considered statistically significant.

Results

Skeletal Myoblasts Genetically Engineered to Express Nuclear \( \beta \)-Gal

To identify grafted skeletal myoblasts from native heart cells, L6 cells were genetically labeled to express \( \beta \)-gal in the nucleus.\(^{14} \) \( \beta \)-gal staining in vitro demonstrated that all the cells expressed enough nuclear \( \beta \)-gal to be stained (Figure 1A). Western blotting confirmed \( \beta \)-gal expression in the genetically manipulated cells, whereas no \( \beta \)-gal expression was observed in the wild-type L6 cells (Figure 1B). Expression of nuclear-targeted \( \beta \)-gal did not affect myogenic differentiation ability in vitro. In a differentiation medium containing 2% horse serum instead of 10% FCS,\(^{18} \) the genetically labeled L6 myoblasts could be induced to undergo myodif-
fermentation and form multinucleated myotubes (hematoxylin and eosin staining, Figure 1C).

Cell Transplantation to the Heart Through the Coronary Artery

One million β-gal–expressing L6 skeletal myoblasts were infused into the heart through the coronary arteries, followed by a 10-minute incubation under increased intracoronary pressure (50 to 80 mm Hg) and heterotopic heart transplantation. The overall operative mortality rate was ≈6%, mainly related to ileus and intra-abdominal infection.

Functional Assessment of Hearts After Cell Transplantation

Functional assessment was performed to assess the myocardial damage caused by the cell transplantation procedure. At no selected time point after cell transplantation did we observe any functional reduction in cell-transplanted hearts as compared with control-treated hearts in terms of heart rate, maximum dP/dt, minimum dP/dt, and coronary flow. The data for day 28 is shown in the Table. Additionally, no dysrhythmic effects caused by infused cells were noted.

Time Course of Graft Cell Survival

Assay for β-gal activity with ONPG was done to evaluate graft cell survival after cell transplantation. First, the standard samples that were created by mixing isolated hearts with a known number (1×10^6, 1×10^5, 5×10^5, or 1×10^4) of β-gal–expressing cells were analyzed. As a result, β-gal activity was 3.4±0.4, 37.2±3.5, 57.8±6.2, 120.6±15.7, or 173.1±20.5 OD_{420}/g protein, respectively. The time course of β-gal activity after cell transplantation is shown in Figure 2. Using the standard scale on the right of the graph, we estimated that ≈9×10^5 cells were present in the heart 10 minutes after infusion, decreasing to ≈5×10^5 cells on day 3. The β-gal–expressing myoblasts quickly increased in number to ≈2.5×10^6 on day 7 with a slow rise between day 7 and day 28, finally up to 5×10^6 cells. The values for the control hearts were <10 OD_{420}/g protein throughout the postoperative period.

Histological Findings of Engrafted Skeletal Myoblasts

The transplanted hearts were collected at the selected time points and stained for β-gal. Ten minutes after cell transplantation, grafted cells were found throughout the both left and right coronary distributions in all cardiac layers, where they appeared to be entrapped within the lumina of small capillaries, occasionally permeating into the myocardial interstitium (Figure 3). At day 28 after cell transplantation, under a low-power magnification, discrete positively stained loci were observed to be widely distributed throughout the cardiac layers of left and right coronary territories (Figure 4A). At some of these loci, surviving cells formed colonies composed of the β-gal–positive myoblasts that appeared to be undifferentiated (Figure 4B). It was, in contrast, observed that at other loci, surviving myoblasts had completely differentiated into β-gal–positive multinucleated myotubes that aligned with the cardiac fiber axis and had integrated into the native myocardium (Figure 4C). Histological evidence of myocardial thrombosis or infarction was not identified.

Expression of Cx43

Cardiomyocytes are electrically coupled to adjacent cells by specialized gap junctions, composed of the hexamers of the
widely in the heart. Taylor and coworkers suggested the cavity and evaluated that 50,000 myoblasts were entrapped of skeletal myoblasts by injection into the mouse left ventricular cells were detected 1 week after engraftment. The translo-
efficiency, however, was not adequate: Only 700 surviving myoblasts into the rabbit heart. The grafting possibility of catheter-associated selective coronary infusion and colleagues examined an arterial delivery method of cells globally into the myocardium. Previously, Robinson al ) previously reported on a system for intracoronary plasty. It is advantageous not only in producing less myocar-
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protein Cx43, which allow the exchange of ions and small molecules between adjacent cells. A linear pattern of staining for Cx43 at apparent cardiomyocyte-cardiomyocyte interfaces was observed throughout myocardium. Additionally, we identified Cx43 expression at the interfaces between skeletal muscle-derived cells forming myotubes and native cardiomyocytes, as shown in Figure 4D.

Discussion

Intracoronary infusion of cells provides theoretical merits over direct intramuscular injection in cellular cardiomyo-
plasty. It is advantageous not only in producing less myocardial damage during engraftment but also in disseminating cells globally into the myocardium. Previously, Robinson and colleagues examined an arterial delivery method of skeletal myoblasts by injection into the mouse left ventricular cavity and evaluated that 50,000 myoblasts were entrapped widely in the heart. Taylor and coworkers suggested the possibility of catheter-associated selective coronary infusion of skeletal myoblasts into the rabbit heart. The grafting efficiency, however, was not adequate: Only 700 surviving cells were detected 1 week after engraftment. The translocation of entrapped cells from coronary capillaries to the myocardial interstitium can be postulated to be a major limiting factor of graft survival. Willerson’s group demonstrated that 90% of the total number of 1 million myoblasts flushed into coronary capillaries or had migrated into the myocardial interstitium at day 0. This observation was supported by our unpublished data that the number of myoblasts flushed into coronary effluent before heart transplantation was only 3% to 5% of the.

Figure 3. Histological findings of grafted skeletal myoblasts at day 0. One million β-gal-expressing L6 cells were injected into heart through coronary artery. At day 0 (after 10-minute incubation without heart transplantation), grafted cells were found throughout both coronary distributions in all cardiac layers, where they appeared to be entrapped within small capillaries, occasionally permeating into myocardial interstitium (A, magnification ×100; C, magnification ×400; D, magnification ×400). Control hearts showed no positive staining (B, magnification ×100).

Figure 4. Histological findings of grafted skeletal myoblasts at day 28. At day 28 of cell transplantation through coronary artery, β-gal-positive L6 cells were detected widely at discrete loci in myocardium (A, magnification ×100). At some loci, they formed colonies composed of undifferentiated β-gal-positive myoblasts (B, magnification ×400). At other loci, in contrast, surviving myoblasts had differentiated into multinucleated myotubes and native cardiomyocytes (arrowheads; D, magnification ×800). Nuclei of skeletal muscle-derived myotubes are stained for β-gal, whereas cardiomyocyte nuclei are unstained (arrows).
initial number of cells infused. The graft cell survival was \( \approx 50\% \) on day 3. Subsequently, the \( \beta\text{-gal} \)–expressing cells quickly increased in number to \( \approx 2 \times 10^6 \) on day 7, with a slow rise from day 7, finally up to \( 5 \times 10^6 \) on day 28. Discrete loci of grafted cells were observed to be widely distributed throughout cardiac layers of both left and right coronary territories on day 28. No histological evidence of large myocardial infarction or functional reduction was identified after cell transplantation in any hearts analyzed. No \( \beta\text{-gal} \)–stained cells were observed in the liver, kidney, spleen, and lung after cell transplantation (data not shown). We therefore consider that this cell-grafting system could disseminate cells globally into the myocardium with reasonable success. This system for intracoronary infusion followed by heterotopic heart transplantation would be relevant to a clinical situation in which the failing heart is arrested by cardioplegic solution and infused with cells through the intracoronary route with coronary sinus occlusion followed by circulatory support with a ventricular assist device until heart function has improved.

In the present study, the function of the treated normal heart was not improved. Although one might expect that infusion of a larger number of skeletal myoblasts might improve function of the normal heart, it is not known how many cells would be required to achieve this. In addition, improvement of cardiac function after skeletal myoblast transplantation is affected not only by cell number infused but also by many factors such as the survival, proliferation, integration, and differentiation of grafted cells. The main aim of our study was to clarify the feasibility of intracoronary cell transplantation and to investigate the behavior of infused skeletal myoblasts. We therefore used normal hearts as myoblast recipients and have demonstrated that this method did not damage cardiac function. We shall study whether the number of skeletal myoblasts infused with the present method is enough to improve the damaged heart’s function by using some heart failure models in future work.

As a semiquantitative indicator of cell number surviving in the myocardium, we used \( \beta\text{-gal} \) activity. Stably transfected clonal cells can generally be expected to express \( \beta\text{-gal} \) constantly on the whole for an extended period, though the level of expression of an individual cell may be affected by cell cycle and circumstance to some extent. Therefore, one can expect the \( \beta\text{-gal} \) activity to be directly proportional to the number of the cells (proliferation).\(^{14,15}\) Using this measurement, we observed that the number of \( \beta\text{-gal} \)–expressing cells existing in the heart rapidly increased up to day 7, with a gradual rise thereafter. This observation suggested that the surviving myoblasts proliferated in the early period and began to differentiate on reaching a particular cellular concentration. The mechanism of switching from the proliferation to the differentiation state with cell cycle withdrawal is likely to involve cyclin-dependent kinases and their inhibitors,\(^{22,23}\) which can be modulated by certain growth factors.\(^{24}\)

Further study, however, is necessary to clarify this issue. We have also shown in these experiments that surviving \( \beta\text{-gal} \)–positive myoblasts behaved in two different ways: Some myoblasts fully differentiated into multinucleated myotubes and integrated into the native myocardium; others formed undifferentiated colonies. It is unknown why some remained in an undifferentiated state whereas others differentiated. Even though the differentiation ability of the \( \beta\text{-gal} \)–expressing myoblasts was observed in vitro, it might not be sufficient when transplanted into the myocardium in vivo. To solve this problem, it would be useful to enhance differentiation potential by genetically engineering grafted myoblasts to overexpress certain beneficial proteins such as Cx43.\(^{25}\)

Intercellular communication between grafted myoblasts and native cardiomyocytes is another concern in skeletal myoblast transplantation to the heart. Cardiac cells are electrically coupled to adjacent cells by specialized gap junctions, composed of hexamers of the protein Cx43.\(^{19,20,25}\) Exchange of ions and small molecules between cells also occurs across these junctions. Gap junctions are found within the intercalated disk and at sites of side-to-side contact between cardiac cells.\(^{19,20}\) It still remains controversial whether grafted skeletal myoblasts could form sufficient gap junctions with native cardiomyocytes.\(^{3,4,8,9}\) We identified Cx43 expression at the interfaces between skeletal muscle–derived cells forming myotubes and native cardiomyocytes, suggesting gap junction formation between these cells.

In conclusion, we have developed a novel method for efficient cell transplantation to the heart by using intracoronary infusion. This method, which is applicable to the clinical setting in cardiac surgery, could be useful to globally disseminate cells into the heart with little myocardial damage.

References

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