



Effectiveness of Transient Immunosuppression Using Cyclosporine for Xenomyoblast Transplantation for Cardiac Repair

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ABSTRACT

We studied the survival of human myoblast for cellular myocardial reconstruction in a porcine model of chronic myocardial ischemia with immune tolerance using transient immunosuppression. A porcine model of chronic cardiac ischemia was created in 10 pigs (DMEM medium-injected $n = 4$; myoblast transplanted $n = 6$) by clamping ameroid ring around left circumflex coronary artery. Three weeks later, 3×10^8 human myoblasts carrying *lac-z* reporter gene were transplanted in multiple sites (0.25 mL each) into the left ventricular wall. Immunosuppression was achieved with 5 mg/kg cyclosporine for 6 weeks after cell transplantation. After animals were euthanized between 6 and 30 weeks after cell transplantation; the heart was removed for histological studies.

Discontinuation of immunosuppression after 6 weeks of cell transplantation did not result in donor cell rejection. The *lac-z*-positive donor cells were detected in porcine host cardiac tissue for up to 30 weeks posttransplantation, expressing human skeletal myosin heavy chain. The results highlight the effectiveness of transient immunosuppression for myoblast transplantation for cardiac repair.

SEVERAL PRECLINICAL STUDIES and phase I human clinical trials have demonstrated that myoblast transplantation leads to improved function of a failing heart.¹⁻³ This cell-based approach supplements the inadequate intrinsic repair mechanisms of the myocardium, regenerating new muscle to replace the dysfunctional, necrotic cardiomyocytes.^{4,5} Myoblasts from autologous, allogenic, xenogenic, or established cell lines have been used.⁶⁻⁸ Transplantation of myoblasts from other than an autologous source is problematic since it should result in rejection of the cell graft.⁹ Cultured myoblasts show extensive cell death even when transplanted into an autologous host.¹⁰ The rejection process may be mediated by multiple factors including cellular and humoral host immune responses, complement activation, cytokines and growth factors.^{11,12} With the emerging interest in the use of cells and organs from nonhuman sources, cell transplantation protocols need to be optimized to provide insight into the in vivo behavior of transplanted donor myoblasts. We report a porcine heart model of chronic ischemia with immunotolerance of xenotransplanted human myoblasts following transient immunosuppression. Well-differentiated donor-derived muscle tissue was detected up to 30 weeks after transplantation. These results suggest that this porcine model for xenomyoblast transplantation provides novel

opportunities for human muscle research in cardiovascular therapeutics.

MATERIALS AND METHODS

Human Myoblast Culture and Labeling

Human myoblasts (Cell Transplantation Inc, Singapore) were expanded in collagen-coated tissue culture flasks at 37°C in 5% CO₂. The purity of the myoblast culture was assessed by desmin expression using an anti-human desmin-specific immunostaining kit (Sigma, USA). The cells labeled with bromodeoxyanidine (BrdU) were transduced with a replication-defective, retroviral vector carrying *lac-z* reporter gene with a nuclear localization signal for posttransplant identification. The transduction efficiency was

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confirmed by 5-bromo-4-chloro-3-indoyl- β -D-galactoside (X-gal) staining for *lac-z* expression.

Staining for β -Galactosidase Expression

The *lac-z*-transduced myoblasts were fixed with 0.5% glutaraldehyde for 15 minutes at room temperature. After rinsing with PBS containing 1 mmol/L MgCl₂, the cells were incubated at 37°C overnight with X-gal (Sigma, USA; 40 mg/mL in dimethylformamide) in X-gal buffer containing 35 mmol/L each of potassium ferricyanide and potassium ferrocyanide, 2 mmol/L magnesium chloride, and 0.1% sodium dodecyl sulfate in PBS. After rinsing in PBS the cells were observed under a microscope.

Development of Animal Model

All animal procedures were performed in accordance with the institutional protocols and guidelines approved by National University of Singapore and assisted by a veterinary doctor. The surgical manipulations were conducted under anesthesia with continuous electrocardiographic monitoring.

A chronic ischemia model was performed in 10 adult female Yorkshire swine, each weighing 30 ± 5 kg: group 1 of six animals was myoblast transplanted while the group 2 four hosts were injected with basal DMEM medium without myoblasts. All animals were maintained on cyclosporine. The hosts were anesthetized using 2% isoflurane inhalation and 100% oxygen (2 L/min) and kept on mechanical ventilation. An IV line provided access for saline infusion throughout the procedure. The heart was exposed by a limited left side thoracotomy and a 2.0-mm internal diameter ameroid steel ring (Research Instrumentation, SW, USA) was placed around the left circumflex artery at its most proximal position. The animal was maintained on cephalosporin for 4 to 5 days postoperatively for prevention of infection. The success of the ischemic model was confirmed by Tc^{99m} -MIBI nuclear imaging.

Myoblast Transplantation and Posttransplantation Animal Care

Three weeks after preparation of the model the animal underwent a second left sided thoracotomy. A myoblast suspension in 5 mL basal DMEM medium containing 3×10^8 cells was injected at 20 different sites (0.25 mL per injection site) intramyocardially in group 1 animals. The control group 2 animals were injected with 5 mL basal DMEM medium without cells. Immunosuppression was provided by cyclosporine (5 mg/kg) beginning 5 days prior to and continuing to 6 weeks after transplantation. Cephalosporin (40 mg/kg) was administered to all animals postoperatively for the first week.

The animals were euthanized between 6 and 30 weeks after myoblast transplantation. The heart was excised and the marked area of left ventricle chopped into 5-mm³ pieces and either prepared in 10% formalin or frozen in liquid nitrogen-cooled isopentane for histochemical and immunohistochemical studies, respectively.

Histochemical and Immunohistochemical Studies

Sections of 6 to 8 μ m cut from frozen slices were stained for β -gal expression. Hematoxylin and eosin staining was performed to visualize the muscle. Masson trichome staining was performed to delineate fibrous from normal tissue. Sections positive for β -gal expression were immunostained for the expression of the slow

isoform of human skeletal muscle myosin heavy chain (Chemicon Int, USA), and the fast isoform of human skeletal actin human MHC class I and MHC class II (NeoMarkers, USA), as well as porcine-specific CD3 ϵ and monocytes (BD Pharmingen, USA) according to the manufacturer's instructions.

RESULTS

Myoblast Culture and Transplantation

The adherent monolayer culture of myoblasts showed 98% purity for desmin expression with 99% viability. More than 75% to 80% of cells were positive for β -gal expression in vitro.

Histochemical and Immunohistochemical Studies

Histochemical studies for β -gal⁺ positive myoblasts in the host tissue showed extensive donor cell survival in the group 1 animals, a finding that was confirmed by immunohistochemistry for BrdU. Donor cell nuclei were not haphazard in arrangement; rather, they were aligned in accordance with the host tissue organization. The extent of β -gal expression was variable and nonhomogenous between the regions injected versus those not injected with myoblasts. The samples of myocardium from group 2 were without BrdU or *lac-z* activity. The *lac-z*-positive cardiac tissue sections also expressed human skeletal muscle myosin and actin.

Transient Immunosuppression and Myoblast Survival

An interesting feature of our study was the induction of long-term host acceptance of the xenografted myoblasts despite the limited immunosuppressive treatment (Figure 1). The CsA regimen (5 mg/kg starting 5 days prior to and until 6 weeks posttransplantation) effectively contributed toward the donor cell survival. At the site of the graft we did not observe infiltration of the porcine cardiac tissue sections with cells positive for CD4⁺ or CD8⁺, with monocytes or with granulocytes, indicating successful immunosuppression. Furthermore, immunostaining of porcine cardiac tissue for human MHC-I and II expression (using human myoblasts and human leukocytes as positive controls) failed to reveal MHC class I expression at 6 weeks posttransplantation.

DISCUSSION

The phase I studies¹³ suggest cellular xenotransplantation holds great clinical promise. Efficient, functional cardiac repair by myoblast transplantation in humans requires extensive characterization of the in vivo behavior of human myoblasts. This requires an animal model in which xenografts are tolerated for long periods of time. The pig is the first choice as a xenotransplantation model, due to its close physiological proximity to humans. In view of the difficulties in the use of human subjects to study graft behavior, we developed a pig heart model.

One basic limitation encountered in myoblast transplan-

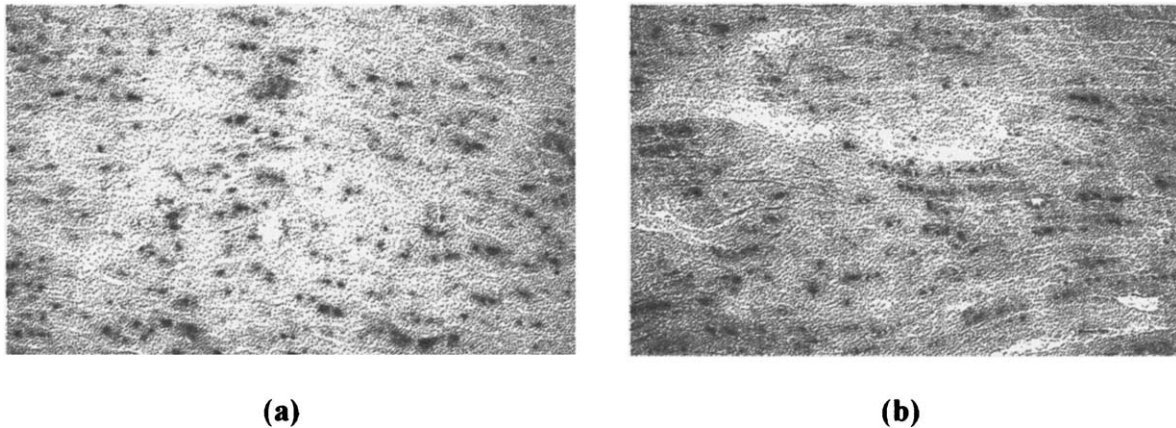


Fig 1. *Lac-z*-expressing human myoblasts in the porcine heart tissue at **(a)** 6 weeks and **(b)** 30 weeks posttransplantation. Immunosuppression was discontinued after 6 weeks of myoblast transplantation; however, the xenograft survived without any sign of rejection.

tation therapy is the rapid donor cell death and rejection. Humoral and cellular immune reactions are responsible for the poor outcome of myoblast transplantations, especially allo- and xenografts.^{14–16} Most previous studies of myoblast transplant used immunosuppressive therapy to enhance cell acceptance and survival. The success of myoblast transplantation varied with the effectiveness of the immunosuppression.^{17–19} During the present study, we found that cyclosporine (5 mg/kg starting 5 days prior to and until 6 weeks after myoblast transplantation) prevented donor cell rejection. Of particular interest and significance is our observation that discontinuation of immunosuppressive treatment at 6 weeks after cell injection did not affect donor cell survival in the host tissue. Similar results have also been reported by others for allo- and xenomyoblast transplantation.^{17,20} The unresponsiveness of the host immune system toward the presence of a xenograft raises the intriguing possibility that human myoblasts enjoy a “conditionally immunoprivileged” status in porcine myocardium. The use of cyclosporine during the crucial early phase when myoblast express MHC molecules may set conditions conducive for myoblasts to escape the host immune response. Once the donor cells have differentiated into myotubes and myofibers, they down-regulate the expression of MHC molecules and are accepted as “self” by the host immune system. The poor survival of donor cells has also been related more to the contaminant populations in the myoblast preparations, including donor endothelial cells and fibroblasts rather than the myoblast themselves. The myoblasts used in the present study were >98% pure based upon desmin expression.

We conclude that induction of immune tolerance by using cyclosporine in the initial phase during myoblast transplantation improves long-term donor cell survival and may help to reduce the adverse effects associated with sustained immunosuppressive treatment.

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