Long-Term Survival of Xenografted Neonatal Cardiomyocytes by Adenovirus-Mediated CTLA4-Ig Expression and CD40 Blockade

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Background—Prolonged survival of xenografted neonatal cardiomyocytes was achieved by blocking the CD28/B7 costimulatory pathway via CTLA4-Ig gene transfer. We examined the long-term survival of xenografted neonatal cardiomyocytes by adenovirus-mediated CTLA4-Ig expression and transient CD40 blockade with anti-CD40L monoclonal antibody (MR1).

Methods and Results—Neonatal cardiomyocytes derived from Dark Agouti rats were infected with CTLA4-Ig–expressing adenovirus vectors and injected directly into the normal myocardium of C3H/He mice. Mice were also given an intraperitoneal injection of 500 μg MR1 (CTLA+MR group, n=30) or control immunoglobulin (CTLA group, n=30) 1 hour before and 1, 3, and 7 days after cardiomyocyte implantation. As a control, cells infected with β-Gal–expressing adenovirus vector (RL group, n=15) and cells without infection (control group, n=15) were injected into additional mice. Mice from all groups were killed 2, 4, and 8 weeks after xenotransplantation, and mice from the CTLA+MR and CTLA groups were killed 4 and 6 months after xenotransplantation. Neonatal cardiomyocytes were successfully infected by adenovirus vectors. Immunohistochemical analysis showed that the xenografted cardiomyocytes survived and expressed CTLA4-Ig for 6 months in all mice from the CTLA+MR and CTLA groups. A gap junction between the xenografted and host cardiomyocytes was also confirmed. Conversely, neonatal cardiomyocytes did not survive for even 2 weeks after xenotransplantation in the mice from the RL and control groups.

Conclusions—Long-term survival of xenografted neonatal cardiomyocytes was achieved by adenovirus-mediated CTLA4-Ig expression and transient CD40 blockade. (Circulation. 2003;108:1760-1765.)

Key Words: myocardium □ transplantation □ survival

Although mature cardiomyocytes have a limited ability to proliferate after injury,1 the regeneration of lost cardiomyocytes is generally very poor. Cell transplantation is a potential method of repairing injured myocardium, for which several cell sources, including fetal cardiomyocytes, myoblasts, bone marrow cells, and others, have been used.2–10 Experimental studies have shown the effectiveness and feasibility of transplanting these cells into injured myocardium. On the basis of the electrophysiological characteristics of myocardium, fetal or neonatal cardiomyocytes are considered among the most ideal cell sources for repairing injured myocardium. To date, allografts of fetal or neonatal cardiomyocytes are the best-documented and most successful method of repairing injured myocardium. Allografted fetal or neonatal cardiomyocytes have shown long-term survival, differentiation and maturation, gap junction formation with host cardiomyocytes, and improved heart function.2–10

Despite their success, allografts of fetal cardiomyocytes may not be clinically feasible, first, because of their limited supply, and second, for ethical reasons. However, xenografts of fetal cardiomyocytes might be considered as an alternative if the problem of immunologic rejection could be overcome. A combined blockade of the CD40 and CD28 pathways has been shown to prolong the survival of xenografts,11–17 and we have observed prolonged survival of xenografted neonatal cardiomyocytes via adenovirus-mediated CTLA4-Ig expression for up to 8 weeks.18

In the present study, we transplanted rat neonatal cardiomyocytes into the myocardium of mice by combined blockade of the CD28 pathway via adenovirus-mediated CTLA4-Ig expression and of the CD40 pathway, with the transient administration of anti-CD40L monoclonal antibody (MR1). This report describes the long-term survival of xenografted neonatal cardiomyocytes for up to 6 months of follow-up.

Methods

Experimental Animals
We used neonatal Dark Agouti (DA) rats and 12-week-old C3/He mice (Japan SLC, Inc, Hamamatsu, Shizuoka, Japan) in this study, which was approved by the Institutional Animal Care and Use
Committee of Yamaguchi University School of Medicine. Animals were bred in clean laboratory conditions with free access to food and water in a temperature-controlled environment under a 12-hour light-dark cycle.

Isolation and Culture of Neonatal Cardiomyocytes
Cardiomyocytes were derived from newborn DA rats within 24 hours of birth. Cells were isolated by use of the neonatal cardiomyocyte isolation system (Worthington Biochemical Corp) according to the manufacturer’s instructions. Cardiomyocytes were then cultured in Leibovitz medium (L-15) supplemented with 10% FBS (Gibco), 100 mg/mL penicillin, and 250 mg/mL streptomycin (Gibco) in a humidified atmosphere with 5% CO₂ at 37°C.

Adenoviral Infection of Neonatal Cardiomyocytes
Recombinant adenovirus vectors carrying CTLA4-Ig (AdCTLA) or β-galactosidase (AdRL) were prepared as described previously. Viral titers of AdCTLA and AdRL were 2.8×10⁸ and 2.5×10⁸ pfu/mL, respectively. Fresh suspended rat cardiomyocytes (3×10⁶ cells/mL) were seeded in a culture dish, and AdCTLA or AdRL vectors were then added at various concentrations according to the multiplicity of infection (MOI); namely, 1000, 500, 200, 100, 50, or 10. After 1 hour of incubation, nonadherent cells were collected and washed 3 times with L-15, then cultured as described above. To test the expression efficiency, cells were fixed in 0.5% glutaraldehyde for 10 minutes after 1, 2, and 3 days of culture, then X-gal–stained or immunohistochemically stained with FITC-labeled anti-CTLA4-Ig antibody (Southern Biotechnology Associates, Inc).

The cardiomyocytes used for xenografts were infected with AdCTLA or AdRL at MOI 50 as described. After 1 hour of infection, cells were collected and resuspended at a concentration of 5×10⁷ cells/mL for immediate transplantation.

Cell Transplantation
The rat neonatal cardiomyocytes with AdCTLA infection were transplanted into the myocardium of normal left ventricular walls in C3H/He mice as described previously. Briefly, mice were given general anesthesia, and tracheal intubation was performed with a 24F intravascular catheter. A mouse ventilator (model 687, Harvard Apparatus, Inc) was used to ventilate the animals with room air at a tidal volume of 10 mL/kg at 80 respirations/min. A left thoracotomy incision was made through the fourth intercostal space, and the thoracic cavity was opened. A 30-gauge needle attached to a 100-μL microsyringe was inserted into the left ventricle of the heart; 5 μL of troponin-I antibody (Caltag Laboratories) was then used to detect expression of ANP. Because the expression of ANP was negative in rat neonatal cardiomyocytes 4 months after transplantation, the long-term survival of xenografted cardiomyocytes was assessed by the expression of troponin-I after immunostaining with the rabbit anti-rat troponin-I antibody, which specifically labeled the cardiomyocytes of rats but not mice. The survival of xenografted cardiomyocytes was also estimated quantitatively by counting the number of positive cells per field under fluorescence microscopy with 400-fold magnification. The percentage of positive cells per field was used for statistical analysis. X-gal staining was also performed in the RL group.

To observe the formation of the cell junction between the xenografted and host cardiomyocytes 6 months after transplantation, the surviving cardiomyocytes were labeled by immunostaining with the rabbit anti-rat cytokeratin antibody and then immunostained with the mouse anti-rat cardiac troponin-I antibody. The gap junction was then stained with rat anti-rabbit conjugate and visualized by fluorescence microscopy.

Statistical Analysis
Data are presented as mean±SD. Statistical significance was evaluated by ANOVA followed by Scheffe’s procedure and by repeated ANOVA to test for interactions. A value of P<0.05 was considered significant.

Results
In Vivo Adenovirus-Mediated Gene Transfer of Neonatal Cardiomyocytes
The percentages of neonatal cardiomyocytes infected with adenoviral vectors are shown in Figure 1. X-gal staining and immunostaining showed an expression efficiency of ~60% at MOI 50 to 200 after infection with AdCTLA or AdRL. The cytotoxicity of the cardiomyocytes was not significant 3 days after the application of adenovirus at MOI<100; however, ~20%, 40%, and 50% of the cardiomyocytes died within 3 days of application at MOIs of 200, 500, and 1000, respectively, suggesting that MOI 50 is suitable for neonatal cardiomyocyte infection by the adenovirus vector. Therefore, we selected MOI 50 to infect the neonatal cardiomyocytes in our xenotransplantation study.

Local Infiltration of Lymphocytes After the Xenotransplantation of Cardiomyocytes
The local infiltration of CD45, CD8, and CD4 in the myocardium of mice is shown in Figure 2. There was significantly less infiltration of CD45 cells in the CTLA and CTLA+MR groups than in the RL and control groups (Figure 2A). Quantitative analysis showed that the numbers of CD45, CD8, and CD4 cells were significantly lower in the CTLA and CTLA+MR groups than in the RL and control groups at 2 and 4 weeks (Figure 2B, P<0.05) but not 8 weeks after the xenotransplantation of cardiomyocytes. The numbers of CD45, CD8, and CD4 cells in the CTLA+MR group were...
also significantly lower than those in the CTLA group 2 weeks after the xenotransplantation of cardiomyocytes (Figure 2B, \( P < 0.05 \)).

**CTLA4-Ig Expression in Neonatal Cardiomyocytes After Xenotransplantation**

Immunostaining analysis showed that some cells in the normal myocardium expressed CTLA4-Ig positively for up to 6 months after the xenotransplantation of cardiomyocytes in both the CTLA and CTLA + MR groups (Figure 3). However, the number of CTLA4-Ig-positive cells decreased with time after the xenotransplantation, and the expression of CTLA-Ig was dimmer 4 and 6 months later than in the first 8 weeks. These findings indicated that CTLA4-Ig was produced by the xenografted cells for up to 6 months but tended to decrease with time after transplantation.

**Survival and Maturation of Neonatal Cardiomyocytes After Xenotransplantation Into Mice**

We found that some cells were stained positively by the anti-ANP antibody, which was expressed specifically in fetal and neonatal but not in adult cardiomyocytes, in all mice from the CTLA and CTLA + MR groups up to 8 weeks after the xenografting of rat neonatal cardiomyocytes. This demonstrates the survival of xenografted rat neonatal cardiomyocytes. The majority of these ANP-positive cells also expressed CTLA4-Ig simultaneously. However, no surviving rat neonatal cardiomyocytes were detected in the myocardium of mice from either the RL or control groups 2 weeks after the xenografting of rat neonatal cardiomyocytes.

We did not find any ANP-positive cells in mice from the CTLA and CTLA + MR groups 4 or 6 months after xenotransplantation, although some of the CTLA4-Ig-positive cells were still detectable. After we changed the antibody of troponin-I for labeling the survival of xenografted rat neonatal cardiomyocytes 4 and 6 months later, we found that some cells showed positivity to troponin-I and that the majority also expressed CTLA4-Ig. These cells showed morphological differentiation and maturation (Figure 4, B–D) similar to adult cardiomyocytes, and some joined to form cell islands (Figure 4A) within the mouse myocardium 6 months after transplantation. This finding suggested that these grafted rat neonatal cardiomyocytes were still surviving even 6 months later and matured with time after transplantation. Quantitative estimation showed that the survival of xenografted cardiomyocytes was significantly higher in the CTLA + MR group than in the CTLA group (Figure 4E, \( P < 0.01 \)).

**Cell Junction Between Xenografted and Host Cardiomyocytes**

Immunostaining analysis showed that the gap junction was regular and distinct in the normal myocardium of mice but
irregular within the xenografted cardiomyocytes (Figure 5B). However, the formation of a gap junction between xenografted and host cardiomyocytes was also found 6 months after cell transplantation (Figure 5C). No arrhythmia or other significant abnormal findings were recorded on ECGs in any of the mice 4 and 6 months after cardiomyocyte xenotransplantation (Figure 6), also suggesting the formation of cell junctions between xenografted and host cardiomyocytes.

**Discussion**

Although cell transplantation is considered to be a feasible and effective method for repairing injured myocardium, the selection of cell sources for transplantation remains a subject of controversy. An ideal cell source for repairing injured myocardium would (1) provide sufficient cells that could be collected easily; (2) have the same electrophysiological characteristics as cardiomyocytes; (3) have no immunologic or ethical problems for clinical application; and (4) survive efficiently for a long time, form a cell junction, and keep a synchronously beating network with host cardiomyocytes after transplantation.

To date, several cell sources have been assessed in experimental investigations or clinical trials, but one or more problems must still be overcome. Heterogenic neonatal cardiomyocytes should also be considered as a cell source for myocardium repair if the problem of immunologic rejection could be resolved after transplantation.

Combined blockade of the CD40 and CD28 pathways provided a possible means of resolving the problem of immunologic rejection of xenografts, and in our previous study, we observed the prolonged survival of xenografted neonatal cardiomyocytes via adenovirus-mediated CTLA4-Ig expression for 8 weeks. In this study, we blocked the CD28/B7 costimulatory pathway by adenovirus-mediated CTLA4-Ig expression and the CD154/CD40 costimulatory pathway by the transient administration of MR1 antibody. The CTLA4-Ig gene was transferred efficiently into the rat neonatal cardiomyocytes by use of the adenovirus vector.
without significant cytotoxicity. Although the expression of CTLA4-Ig seemed to decrease with time, expression of CTLA4-Ig was observed in these xenografted rat neonatal cardiomyocytes for up to 6 months. This finding suggests that adenovirus-mediated CTLA4-Ig expression is a feasible method for blocking the CD28/B7 costimulatory pathway for a prolonged period.

Previous studies have shown that adenovirus-mediated CTLA4-Ig expression does not cause a significant decrease in local leukocyte infiltration within the first few days after transplantation, but it does decrease thereafter. In the present study, we assessed the local infiltration of lymphocytes >2 weeks after the xenotransplantation of cardiomyocytes. We found that the infiltration of CD45, CD8, and CD4-positive cells was significantly depressed by the blockade of the CD28 pathway via adenovirus-mediated CTLA4-Ig expression and further depressed by the simultaneous blockade of the CD40 pathway via the transient administration of MR1 at 2 and 4 weeks after xenotransplantation. This indicates that the combined blockade of the CD40 and CD28 pathways provided an effective method of inhibiting the immunologic rejection of the xenografts of cardiomyocytes. However, the lymphocyte infiltration decreased rapidly with time in the RL and control groups, with no significant difference found between these groups by 8 weeks after xenotransplantation. This could be explained by the fact that no xenografted cells survived for ≥2 weeks in the RL or control groups.

Although we witnessed prolonged survival of xenografted cardiomyocytes for 8 weeks in our previous study, longer-term survival of grafted cells is critically important for clinical application. In the present study, xenografted rat neonatal cardiomyocytes survived for up to 6 months of follow-up. These surviving rat neonatal cardiomyocytes had the shape of adult-like cardiomyocytes and lost the expression of ANP by 4 and 6 months after xenotransplantation. Our results indicate that the rat neonatal cardiomyocytes survived long term and differentiated into mature cardiomyocytes in the normal myocardium of mice after transplantation. Although long-term survival was also achieved by single CD28 pathway blockade via adenovirus-mediated CTLA4-Ig expression, cell survival was further improved by combination with the transient blockade of the CD40 pathway.

Figure 5. Representative identification of cell junction between xenografted and host cardiomyocytes 6 months after xenotransplantation from mice in CTLA+/MR group (n=8). A. Rat cardiomyocytes (green) survived in mouse myocardium. B. Immunostaining with connexin-43 showed a regular and distinct gap junction (red) in normal myocardium of mice, but gap junction was irregular within xenografted cardiomyocytes. C. Merged double fluorescence showed formation of a gap junction somewhere between xenografted and host cardiomyocytes (arrow).

Figure 6. ECGs of mice in CTLA+/MR group 6 months after cardiomyocyte xenotransplantation (A) and ECGs of normal mice (B). Compared with normal mice, no arrhythmias or other significant abnormal findings were recorded in any mice in CTLA and CTLA+/MR groups 4 and 6 months after cardiomyocyte xenotransplantation.
The formation of a gap junction between grafted and host cardiomyocytes means that the engrafted cells will integrate and form electromechanical coupling with the host myocardium, which is critically important for forming a synchronously beating network between the grafted and host cardiomyocytes. In this study, immunostaining with connexin-43 showed a distinct and well-organized gap junction in the host myocardium and a disorganized gap junction among the xenografted rat cardiomyocytes. However, the formation of a gap junction was also seen between the xenografted and host cardiomyocytes. Otherwise, we recorded no abnormal electrocardiography in any mice for up to 6 months after the xenotransplantation of rat neonatal cardiomyocytes. Although further investigation is required, these findings suggest the formation of synchronously beating networks between xenografted and host cardiomyocytes.

Despite these new and remarkable findings, the limitations of this study need to be addressed. First, we did not examine the improvement in cardiac function achieved by the xenotransplantation of neonatal cardiomyocytes. This study was also obviously limited by the fact that we xenografted neonatal cardiomyocytes into normal myocardium. Compared with the normal myocardium, the injured myocardium provided a more severe microenvironment for cell survival and growth, characterized by low tissue blood flow, hypoxia, and impaired nutrition. Cardiomyocytes have shown poor tolerance to ischemic conditions, and conflicting findings have been reported on the influence of survival of allografted cardiomyocytes in injured myocardium compared with normal myocardium. Therefore, it is still unknown whether xenografts of neonatal cardiomyocytes can survive well in injured myocardium with the same therapeutic strategy. We considered that the survival of xenografted neonatal cardiomyocytes in injured myocardium could not present difficulties because the majority of investigations have suggested the survival of allografted cardiomyocytes in injured myocardium. Furthermore, the severe microenvironment in the injured myocardium might be improved by combination therapy, such as inducing angiogenesis to improve blood flow of the injured myocardium.

In conclusion, long-term survival of xenografts of neonatal cardiomyocytes was achieved by creating a blockade of the CD28 pathway via adenosivirus-mediated CTLA4-Ig expression and the transient blockade of the CD40 pathway. We also found the maturation of xenografted neonatal cardiomyocytes and the formation of a gap junction between xenografted and host cardiomyocytes. Our findings suggest that the xenotransplantation of neonatal cardiomyocytes could be an alternative approach for repairing damaged myocardium.

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