Quantitative analysis of survival of transplanted smooth muscle cells with real-time polymerase chain reaction

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Background: Cell transplantation improves heart function after myocardial infarction. This study investigated the survival of implanted cells in normal and infarcted myocardium.

Methods: Male rat aortic smooth muscle cells were cultured. For the in vitro study, male smooth muscle cells mixed with female smooth muscle cells or male smooth muscle cells injected into a piece of female rat myocardium were used to evaluate the accuracy of quantitative real-time polymerase chain reaction to measure Y chromosomes. For the in vivo study, 2 million live or dead male smooth muscle cells were injected into normal or infarcted female myocardium. At 1 hour and 1 and 4 weeks after transplantation, hearts, lungs, and kidneys were harvested for measurement of Y chromosomes.

Results: In vitro, the accuracy of polymerase chain reaction measurement was excellent in cultured cells ($r^2 = 0.996$) and the myocardium ($r^2 = 0.786$). In vivo, 1 hour after 2×10^6 cell implantation, live cell numbers decreased to $1.0 \pm 0.2 \times 10^6$ and $1.1 \pm 0.3 \times 10^6$, and dead cell numbers decreased to $0.9 \pm 0.2 \times 10^6$ and $0.8 \pm 0.2 \times 10^6$ in the normal and infarcted myocardium, respectively ($P < .01$ for all groups). Lungs and kidneys contained 8.5% and 1.5% of the implanted cells, but no cells were detected at 1 week. At 1 week, no dead smooth muscle cells were detected in the normal or infarcted myocardium. The numbers of live cells at 1 and 4 weeks were $0.48 \pm 0.06 \times 10^6$ and $0.27 \pm 0.07 \times 10^6$ in normal myocardium and $0.29 \pm 0.08 \times 10^6$ and $0.18 \pm 0.05 \times 10^6$ in infarcted myocardium.

Conclusions: One hour after implantation, only 50% of smooth muscle cells remained in the implanted area. Some implanted cells deposited in other tissue. Implanted cell survival progressively decreased during the 4-week study.

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Cell transplantation has come of age.¹ Recent experimental and clinical reports have suggested that transplanted cells prevent ventricular dilation and dysfunction.²⁻⁴ The benefits of cell implantation on heart function were proportional to the number of cells injected.⁵ Although a variety of interventions have been proposed to increase cell survival to enhance the functional benefit,^{6,7} techniques to evaluate cell survival in the implanted heart have not been well established.

A variety of methods have been used to evaluate cell survival after myocardial transplantation, including microscopic observation, assays for β -galactosidase activity, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining assay.⁷ Unfortunately, these methods are semiquantitative, and data could not provide precise quantitation of the survival of the implanted cells. Recently, Müller-Ehmsen and associates⁸ used real-time polymerase chain reaction (PCR) targeting the Y chromosome to measure the number of neonatal rat cardiomyocytes that survived transplantation into the female rat myocardium.

In this study we evaluated the accuracy and reproducibility of quantitative real-time PCR to measure the Y chromosome. Then we studied the survival of transplanted male rat smooth muscle cells (SMCs) into normal or infarcted female rat myocardium by using the real-time PCR assay. Lungs, kidneys, and brain were also harvested at different times after cell transplantation to determine whether the implanted cells leaked from the implanted site of the heart.

Materials and Methods

Experimental Animals

Adult male Lewis rats (weighing 250-300 g) were used as cell donors, and female Lewis rats (weighing 200-250 g) were recipients. All procedures were approved by the Animal Care Committee of the Toronto General Research Institute and were performed in compliance with the *Guide to the Care and Use of Experimental Animals* of the Canadian Council on Animal Care and the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (publication no. 85-23; revised 1996).

Preparation of Donor Cells

SMCs were selected for this study because they might be an ideal choice for implantation to stabilize an infarct region and prevent ventricular dilation and dysfunction after a myocardial infarction.^{9,10} Implantation of these cells provided a functional improvement similar to that with fetal cardiomyocytes¹¹ or skeletal muscle myoblasts.¹² We elected to study SMCs transplantation because these cells can be easily harvested from peripheral vessels, they readily proliferate in vitro, they can be cryopreserved, and they respond to hemodynamic stresses with hypertrophy and hyperplasia.

The aorta of male Lewis rats was excised and washed with phosphate-buffered saline (PBS), and the surrounding connective tissue was removed. The endothelial cells were removed with trypsin, and the aorta was minced and incubated in 10 mL of PBS containing 0.2% trypsin (Difco; Invitrogen Corp, Invitrogen Canada Inc, Burlington, Canada), 0.1% type II collagenase (Worthington; Biochemical Corp, Lakewood NJ), and 0.02% glucose for 10 minutes at 37°C. The supernatant containing the isolated cells was transferred into 20 mL of culture medium (Iscove modified Dulbecco medium; Invitrogen Corp) containing 10% fetal bovine serum (Invitrogen Corp), 0.1 mol/L β -mercaptoethanol, 100 U/mL penicillin, and 100 μ g/mL streptomycin. This procedure was repeated 3 times. The cell suspension was then centrifuged at 600g for 5 minutes at room temperature. The cell pellet was resuspended in cultured medium and cultured. The cells were subcultured, and the second-passage cells were used for the studies. Cultured SMCs were identified immunohistochemically by using a monoclonal antibody against α -smooth muscle actin (Sigma-Aldrich, St Louis, Mo) as previously described.^{9,10}

Preparation of Cells Immediately Before Implantation

Live cells. The cultured SMCs were detached by the addition of 0.05% trypsin in PBS to the culture dish for 3 minutes. Then 10 mL of Iscove modified Dulbecco medium without serum was added to the dish, and the cell suspension was centrifuged 580g for 3 minutes. The cell pellet was resuspended at a concentration of $2 \times 10^6/50 \mu\text{L}$ for cell transplantation.

Dead cells. To examine the time required for the adult rat heart to break down the DNA of irreversibly injured male SMCs after transplantation, cultured SMCs were killed by exposing them to 80°C for 5 minutes before cell transplantation. The death of the cells was confirmed by culturing studies.

Labeled cells. To identify the transplanted SMCs in the recipient myocardium, cells were cultured to 50% confluence and then labeled with 5-bromo-2'-deoxyuridine (BrdU; Zymed Lab Inc, South San Francisco, Calif) by adding 25 μL of a 0.4% solution to 10 mL of cultured medium for 24 hours before transplantation, as previously described.^{9,10}

In Vitro Evaluation of Accuracy and Reproducibility

The accuracy and reproducibility of real-time PCR for measuring cell numbers were evaluated by the following methods:

1. The reproducibility of real-time PCR was evaluated with a standard curve.¹³ Genomic DNA of 10^7 SMCs was extracted, and the DNA was dissolved in 200 μL of water. A 4- μL sample corresponding to 10^5 copies of male Y-chromosomal DNA was diluted to produce samples, which had a range from 10^1 to 10^5 copies. Male SMCs isolated from 4 individual rats were used for evaluation of reproducibility. The number of cycles at which fluorescence exceeded the threshold was analyzed by real-time PCR, as described in the next section.
2. The accuracy of the assay and the interference of female cell DNA on the measurement of the male cell counts were evaluated by assaying mixtures of male and female cells.¹⁴ A total of 10^5 cell populations were prepared so that the proportion of male to female cells varied: 0.1%, 1%, 10%,

Figure 1. A, Amplification plots after serial dilutions of standard DNA extracted from male smooth muscle cells (SMCs). In brief, the genomic DNA of 1×10^7 SMCs was extracted and dissolved in 200 μ L of water. DNA samples (2 μ L) were diluted from 1:10 to 1:10⁴. The samples were amplified by real-time PCR. The numbers from 10¹ to 10⁵ correspond to the copy number of male cells. B, Dissociation curves of the PCR products from genomic DNA extracted from male cells. C, Dissociation curves of the PCR product from DNA samples extracted from infarcted female myocardium 4 weeks after male SMC transplantation. The dissociation curves in panel C were similar to those in panel B.

or 100% ($n = 5$ in each group). The number of male cells was then measured with a real-time PCR technique.

- To evaluate the effect of heart tissue and DNA extraction from tissue on accuracy and reproducibility, male cells (0.5 , 1.0 , 2.0 , or 4.0×10^6) were injected into 300 mg of female heart tissue in a test tube ($n = 5$ for each group). The myocardium was immediately frozen in liquid nitrogen. The tissues were ground into a fine powder in a precooled mortar and pestle for DNA extraction. the number of male cells was evaluated with real-time PCR.

Quantitative Analysis of Y-Chromosomal DNA by Real-Time PCR

The DNA of the cells or tissue was extracted with the Qiagen Kit (Qiagen, Mississauga, Ontario, Canada). The DNA was dissolved in 200 μ L of water, and the total amount of DNA was measured by spectrophotometry.

Real-time PCR was performed with SYBR-Green (Applied Biosystems, Foster City, Calif). The SYBR-Green I dye binds to the double-stranded product, resulting in an increase in fluorescence detected by the ABI 7900HT Sequence Detection System

(Applied Biosystems). Figure 1, A illustrates the amplification plot of the target gene in this experimental setting. A specific sequence of rat *Sry3* gene in the Y chromosome was targeted. The genomic DNA taken from male SMCs was used to obtain a standard curve as described previously. The primer pairs (30 nmol/L) were GCA TTT ATG GTG TGG TCC CGC GG and GGC ACT TTA ACC CTT CGA TGA GGC.¹⁵ The cycling conditions were 5 minutes at 50°C, 10 minutes at 95°C for activation of polymerase, and then 30 seconds at 95°C for denaturation, 60 seconds at 62°C inducing annealing, and 30 seconds at 72°C for extension. Forty-five cycles were used. After amplification, dissociation curves were obtained to discriminate between specific and nonspecific products (Figure 1, B and C).

Coronary Artery Ligation

An anterior myocardial infarction was created by ligation of the proximal left anterior descending artery of adult female rats for cell transplantation, as previously described.^{16,17}

Cell Transplantation

Live and dead cells were implanted into normal and infarcted myocardium at 3 weeks after left anterior descending artery ligation. Under general anesthesia, the heart was exposed through a median sternotomy. A purse-string suture was placed around the proposed injection site in the anterior wall of the left ventricle to avoid leakage of cells after injection. Live or dead SMCs ($2 \times 10^6/50 \mu\text{L}$) were injected with an insulin syringe into a single site of the anterior wall near the apex through the purse-string suture. After transplantation, the purse-string suture was tied and left to mark the implantation site. The chest was then closed, and the animals were allowed to recover.

At 1 hour, 1 day, 1 week, and 1 month after cell transplantation, the hearts were harvested. The left ventricular free wall, including the implantation site, was dissected. The samples were frozen in liquid nitrogen and stored at -80°C until DNA extraction, as described previously.

Lungs, brains, and kidneys were harvested 1 hour and 1 week after transplantation. Those tissues were divided to extract DNA in the same manner as with heart. Quantitative analysis of male cells was performed with real-time PCR.

Histology

The heart sections were fixed in 5% glacial acetic acid in methanol, embedded in paraffin, and cut into $10\text{-}\mu\text{m}$ -thick slices. The slices were stained with hematoxylin and eosin as described in the manufacturer specifications (Sigma). Implanted cells at the transplanted region were identified by immunohistochemical staining for BrdU as previously described.^{9,10}

Statistical Analysis

The results were expressed as mean $\pm$ SE. Comparisons between groups were evaluated by a 2-way analysis of variance. When a significant F ratio was obtained, further comparisons were determined by the Bonferroni post hoc test.

Figure 2. The reproducibility of the standard curve obtained by real-time PCR is illustrated. A serial 10-fold dilution of the DNA from the smooth muscle cells was tested 4 times in separate experiments. Each circle corresponds to the result of 1 dilution in 1 assay. The solid line corresponds to the regression analysis ($y = 41.44 - 3.93 \log x$; $r^2 = 0.994$; $P < .001$).

Results

Cell Preparations

Immediately before transplantation, the SMC cultures were stained for α -smooth muscle actin, and the percentage of positive cells was $90\% \pm 3\%$ ($n = 6$). The efficacy of BrdU labeling was $49\% \pm 7\%$ ($n = 6$). The cells that were subjected to lethal injury (80°C for 5 minutes) were examined by light microscopy after culturing for 24 hours after injury, and no cells survived the insult. The cells were spherical and intact but could not attach on the culture dish or proliferate.

Standard Curves for Real-Time PCR

Figure 2 shows the reproducibility of the Y-chromosome quantification by this technique ($n = 4$). The correlation between the number of copies of male cell DNA and the number of cycles detected by fluorescence exceeding the threshold was excellent ($r^2 = 0.994$).

Accuracy of Real-Time PCR

The correlation ($r^2 = 0.996$; $n = 5$) between the number of male cells calculated from the standard curve and the number of male cells added to the female cells was excellent (Figure 3, A). The accuracy of the PCR assay in measuring the number of male cells added into female myocardial tissue was satisfactory. The correlation between the number of male cells calculated from the standard curve and the number of male cells injected into the female myocardium was 0.786 ($n = 5$; Figure 3, B).

Figure 3. A, In vitro assessment of the accuracy of male cell quantification. Male smooth muscle cells (SMCs) were mixed with female SMCs in a percentage varying from 0.1% to 100% in a total of 10^5 cells. Genomic DNA was extracted from the sample, and the number of male cells was determined from the standard curve ($n = 5$). The solid line represents the linear regression analysis ($\log y = 0.99 \log x + 0.03$; $r^2 = 0.996$; $P < .001$). **B, In vitro evaluation of the accuracy of male cell determination.** Male cells ($0.5, 1.0, 2.0$, and 4.0×10^6) were injected into 300 mg of female heart tissue ($n = 5$ each). Genomic DNA was extracted, and the number of male cells was determined from the standard curve. The solid line represents the linear regression analysis ($y = 0.73x + 0.12$; $r^2 = 0.786$; $P < .001$).

Survival of Implanted Cells

Table 1 showed the number of dead cells measured at 1 hour and 1 week after transplantation in normal and infarcted myocardium. Approximately half of the injected cells were detected at the implantation site in both normal and infarcted myocardium 1 hour after transplantation. At 1 week after transplantation, no cells were detected in the transplanted area in either the normal or infarcted region.

Table 2 showed the number of male live cells transplanted into female normal myocardium. Similar to the results obtained with the dead cells at 1 hour after transplantation, only 50% of implanted cells were detected at the implanted area. At 1 or 4 weeks after transplantation, the

TABLE 1. The number of lethally injured male cells transplanted into the heart with time

Recipient female myocardium	Time after transplantation	
	1 h	1 wk
Normal		
No. rats	4	5
No. male cells ($\times 10^6$)	$0.92 \pm 0.18^*$	$0 \pm 0^*$
No. male cells/ 2×10^6 cells (%)	46	0
Infarcted		
No. rats	5	6
No. male cells ($\times 10^6$)	$0.82 \pm 0.22^*$	$0 \pm 0^*$
No. male cells/ 2×10^6 cells (%)	41	0

A total of 2×10^6 male smooth muscle cells were lethally injured by exposing them to 80°C for 5 minutes before transplantation into normal or injured left ventricular free wall. The implanted cell number decreased ($*P < .01$ compared with implanted cell number) by 1 hour and was undetectable at 1 week.

number of cells transplanted into normal and infarcted myocardium decreased ($P < .001$) as compared with the number at 1 hour after transplantation. Although the survival of implanted cells tended to be higher when the cells were transplanted into normal myocardium compared with the numbers in the infarcted myocardium at 1 and 4 weeks after transplantation, the difference was not statistically significant.

Measurement of Implanted Cells in the Lung, Brain, and Kidney

At 1 hour after transplantation, the number of implanted cells was $0.17 \pm 0.10 \times 10^6$ in the lungs, $0.03 \pm 0.03 \times 10^6$ in the kidneys, and none in the brain ($n = 5$ per group). One week after cell transplantation, no cells were found in the lung, kidney, or brain ($n = 3$ per group).

Histology

Four weeks after cell transplantation, the cells were identified in the implanted area of both normal and infarcted myocardium in the live cell transplantation group by staining for BrdU (Figure 4). Mononuclear cell infiltration was present at the transplanted area, indicating a mild immunorejection. In control animals and animals implanted with dead cells, no BrdU staining was detected, and no cell engraftment was found.

Discussion

Cell transplantation has been demonstrated to prevent cardiac dilation and functional deterioration after myocardial infarction in animals⁹⁻¹¹ and in humans.²⁻⁴ The transplanted cells engrafted, induced angiogenesis, and stimulated matrix remodeling. The improvement in regional ventricular function was shown to be proportional to the number of muscle cells injected.⁵ Because few studies have reported the survival of injected cells, an accurate technique to quantify

TABLE 2. The number of live male cells after transplantation into the heart with time

Recipient female myocardium	Time after transplantation		
	1 h	1 wk	4 wk
Normal			
No. rats	5	5	8
No. male cells ($\times 10^6$)	$0.97 \pm 0.23^*$	$0.48 \pm 0.06^{*\dagger}$	$0.27 \pm 0.07^{*\dagger}$
No. male cells/ 2×10^6 cells (%)	49	24	14
Infarcted			
No. rats	5	6	10
No. male cells ($\times 10^6$)	$1.05 \pm 0.27^*$	$0.29 \pm 0.08^{*\dagger}$	$0.18 \pm 0.05^{*\dagger}$
No. male cells/ 2×10^6 cells (%)	53	15	9

A total of 2×10^6 male smooth muscle cells were transplanted into female normal or infarcted left ventricular free wall. The implanted cell numbers decreased ($*P < .01$ for all groups compared with implanted cell number) by 1 hr, 1 wk, and 4 wks.
 $\dagger P < .01$ for all groups as compared with 1 hour after transplantation.

Figure 4. Histologic photomicrographs of the infarcted (A and B) and normal (C and D) myocardium at 4 weeks after smooth muscle cell transplantation. Hematoxylin-eosin (H&E) staining (A and C) of the transplanted myocardium showed the implanted cells. The transplanted cells stained positively for BrdU (arrows in B and D) in the sections adjacent to the H&E sections. Some mononuclear cells (big arrows) were present around the engrafted smooth muscle cells.

survival is necessary. An accurate measurement technique could make a significant contribution to the science of cell transplantation because increasing cell survival might enhance the number of cells engrafting in the infarcted myocardium and might increase the functional recovery after cell transplantation.

Real-time PCR is a promising technique to quantify the number of surviving cells. The technique of quantitative

analysis of the Y chromosome in the female host was applied by Lee and colleagues^{15,18} to evaluate donor male leukocyte survival in female recipients. We used the same primer pairs to target the *Sry3* gene sequence in the rat Y chromosome. We evaluated the method's accuracy, sensitivity, and reproducibility to quantify male muscle cells in female myocardium. As shown in Figure 3, the standard curve was reproducible. The technique was also reliable and

accurately measured a known number of male cells added to either female cells or female heart tissue. The number of cardiomyocytes in the rat ventricle is approximately 120 million.⁸ We used half of the left ventricle for DNA extraction and were able to detect 60,000 cells (3% of the cells initially injected). Theoretically, the minimum number of male cells that could be measured was 10,000 cells, or 0.5% of the cells initially injected. As many as 100 million cells should be detectable on the basis of the dynamic range of the method. The sensitivity of the technique was more than adequate to quantify the number of cells surviving transplantation.

The real-time PCR technique enabled us to measure the number of male cells in female tissue. We found that approximately half of the live or dead cells disappeared within an hour after cell transplantation. Although we attempted to avoid leakage of the cells through the epicardial needle insertion site or into the ventricular cavity, the leakage might have occurred at the injection site, through the needle tracts, as well as into the myocardial venous and lymphatic systems. Because more cells were found in the lungs than in the kidney or in the brain, the cells might have escaped into the cardiac veins and lymphatics rather than into the ventricular cavity. Although the SMCs used in this study did not form tissue in nonmyocardial sites, leakage of cells, such as embryonic or adult stem cells, could result in a viable engraftment.¹⁹ Cell numbers in other organs, such as liver, will need to be examined in future studies.

To accurately evaluate the survival of implanted viable cells, we first investigate the time for the myocardium to eliminate implanted dead cells. Most of the dead cell DNA was removed within the first day after transplantation, and all of the lethally injured cells were completely cleared by 1 week. Therefore, the number of cells surviving 1 and 4 weeks after cell transplantation represented viable engrafted cells rather than the persistence of the DNA of irreversibly injured cells. Our data were in agreement with those reported by Müller-Ehmsen and colleagues,⁸ who injected frozen cells into the hearts.

A substantial number of cells were lost between 1 hour and 1 week after cell transplantation. The loss might be explained by damage to the cell membranes when the cells were injected through the fine needle, by ischemic injury (especially in scar tissue), and by activation of apoptotic pathways.²⁰ If these injuries were an important cause of early cell loss, strategies to prevent cell damage would be expected to improve donor cell survival. The implantation of cells into the middle of an infarct region might also induce early cell attrition because of ischemic or traumatic injury. Previous studies have suggested that cell engraftment was better in the periphery than in the center of an infarct region.²¹ However, muscle cell transplantation has been shown to induce angiogenesis,^{22,23} and this could

facilitate long-term transplanted cell survival. Transfection of donor cells with vascular endothelial growth factor or pretreatment of the ischemic region with fibroblast growth factor before cell transplantation might also enhance transplanted cell viability and functional improvement.^{17,24}

The decrease in transplanted SMC number in both the normal and infarcted myocardium might also be explained by mild immunorejection, because a mild mononuclear infiltration was visible around the engrafted SMCs. Although the Lewis rats were inbred, mild histocompatibility antigen differences exist between male and female individuals.²⁵ The male antigen H-Y may be important in graft rejection or graft-versus-host disease when male cells are transplanted into female organs, which could explain the presence of the mononuclear infiltrate in our study. Future studies are required to determine whether antirejection therapy such as cyclosporine (INN: ciclosporin) would increase cell survival when male cells are transplanted into the female heart.

Our results were similar to those reported by researchers who have estimated cell survival after transplantation. Müller-Ehmsen and associates⁸ found that 23% of neonatal cardiomyocytes survived in the healthy rat myocardium 4 weeks after transplantation. The differences between their results and ours could reflect a variety of factors that influence cell survival, engraftment, and improvement in ventricular function. Neonatal cardiomyocytes could be more resistant to cell injury during implantation and more proliferative than adult SMCs after implantation. The number of cells injected might correlate with the mechanical injury suffered by the cells because of increased pressure in the syringe during injection.

In summary, quantitative analysis of male donor cells with real-time PCR targeting the Y chromosome was a reliable method of evaluating donor cell survival. Significant leakage of the implanted cells occurred and resulted in the cells being trapped in nonmyocardial organs. Large-animal studies using this PCR technique should help to define the optimal conditions for cell transplantation to fulfill the promise of cardiac regeneration.

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