Washout of transplanted cells from the heart: A potential new hurdle for cell transplantation therapy

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Abstract

Objective: The number of viable transplanted cells in the heart is sharply decreased shortly after cell injection. The exact mechanics of cell loss are unclear. We hypothesized that immature cardiac cells transplanted directly into rat heart could be washed out via the cardiac vasculature, and carried to other organs.

Methods: Female Fischer rats were subjected to 60 min of coronary artery occlusion followed by 3 h of reperfusion (OR group) or 4 h or permanent coronary artery occlusion (PO group). Neonatal rat cardiac cells (5 × 10^6) were injected directly into the free wall of the left ventricle at either 15 min post-reperfusion (OR group) or 75 min after occlusion (PO group). At the end of the protocol, a histological analysis for transplanted cells in the heart (i.e. microscopic examination for cells in approximately 790 histologic fields within each heart) and polymerase chain reaction (PCR)-based determination of the Sry gene (a male cell marker) in the heart and other organs were performed.

Results: In the OR group, only 3.39 ± 0.69% fields contained immature cells compared to 6.57 ± 1.33% fields in the PO group (p < 0.05). Cardiac blood vessels contained round, immature cardiomyocytes. PCR analysis revealed that 100% of the animals (5 of 5) in both groups had cells present in their hearts and lungs, 40% of the OR group and 60% of the PO group demonstrated cells in the liver and kidneys, and 40% of the PO group had cells in the spleen.

Conclusion: Neonatal cardiomyocytes injected directly into the area at risk of the heart escape acutely from the infract to other organs through the vascular system of the heart; loss of cells is more prominent with reperfusion.

Keywords: Myocardial infarction; Cell transplantation

Over the last 5 years, the field of cellular transplantation for the treatment of myocardial infarction has blossomed. Numerous studies have utilized various cell types in an attempt to replace scarred myocardium. Fetal and neonatal cardiomyocytes, skeletal muscle myoblasts, hematopoetic, mesenchymal, and embryonic stem cells have all been injected directly into damaged myocardium in an attempt to repair the tissue [1–8]. Several laboratories, including our own, have reported high regenerative capacity of transplanted cells, improvement in left ventricular function, thickening of the scarred wall, and reduced left ventricular dilatation following cell therapy in experimental infarct models [2,5,9]. Invariably, cardiac function in the experimental settings does not completely return to normal.

Jochen Müller-Ehmsen et al., in our group, injected syngeneic neonatal cardiac cells into normal adult rat hearts. At 1 day after transplantation he observed that only 24% of the injected cells were identifiable within the heart [10]. In contrast, when he injected cells into a myocardial infarction induced by coronary occlusion, and devoid of coronary
blood flow, 62% of the cells injected were identifiable [2]. Therefore, we postulated that washout of cells by coronary blood flow might be one potential mechanism by which cells are lost from the heart.

The present study was done to answer the following questions: (1) what effect does either reperfusion or the lack of reperfusion have on the distribution of transplanted cells within the heart? (2) When immature cardiac cells are injected acutely in the myocardium, are they washed out? (3) What is the mechanism if washout does occur? (4) Where do the cells go because of this phenomenon?

1. Materials and methods

The study conforms to the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of Good Samaritan Hospital.

1.1. Surgical procedure

Adult female Fisher CDF rats (Charles River, USA) were used for this study. Rats (n=60) were anesthetized by an intraperitoneal injection of ketamine (75 mg/kg, 100 mg/ml) and xylazine (5 mg/kg, 20 mg/ml). After induction of anesthesia, the neck and chest were shaved, and the animals were placed on a heating pad, intubated and connected to a small animal respirator (Harvard Apparatus, Holliston, Massachusetts; rate of 60 cycles/min, tidal volume of 1 ml/100 g body weight). Additional anesthesia was administered as needed. A neck incision was made and the right carotid artery and left jugular vein were canulated for hemodynamic measurements and drug administration. Next, a left thoracotomy was performed at the fourth intercostal space. After removing the pericardium to expose the heart, a 4-0 suture was placed around the proximal portion of the left coronary artery and threaded through a small plastic tube to create a snare for coronary artery occlusion. After a stabilization period of 5 min, heart rate, blood pressure and temperature were recorded (ADInstruments, Castle Hill, New South Wales, Australia). Animals were then randomized to either the occlusion reperfusion (OR) cell-treated group or permanently occluded (PO) cell-treated group. To create the occlusion, the suture was threaded through the tubing and the tubing was pressed against the coronary artery. The suture was then pulled tight to occlude the artery and a clamp was placed on the suture at the distal end of the tube to keep it secure. The OR group was subjected to 60 min of coronary artery occlusion followed by 3 h of reperfusion; reperfusion was induced by removing the clamp and gently rubbing the artery with a swab to help induce reflow. In the PO group, the snare remained tight against the artery throughout the 4-h period. Hemodynamics were recorded at baseline, 59 min into the occlusion, and 179 min into reperfusion (OR group), or at 239 min into the occlusion (PO group). A few rats received media only as a negative control for the polymerase chain reaction (PCR) analysis.

1.2. Isolation of neonatal cardiomyocytes

Cardiomyocytes were harvested from neonatal Fisher CDF rats (1–3 days) by digestion with collagenase II (95 U/ml) and pancreatin (0.6 mg/ml) in HEPES-phosphate buffer (pH7.4). Isolated cardiac cells were pre-plated at 37 °C for 30 min to reduce the number of non-cardiomyocytes) in Iscove’s Modified Dulbecco’s Medium (IMDM) containing 10% horse serum, 5% bovine serum, 100 mg/ml of streptomycin and 100 U/ml of penicillin G. Cells were then re-plated with media and incubated at 37 °C, in 95% O₂+5% CO₂ for 1–2 days until needed.

1.3. Transplantation of cells

Fifteen minutes after releasing the snare in the OR group, and 75 min after occlusion in the PO group, 5 × 10⁶ neonatal cardiac cells in total volume of 50–75 μl of media were injected directly into the left ventricle free wall below the snare, using a tuberculin syringe with the needle bent at approximately 45°. Media injections were also performed in this manner. Starting near the apex of the heart, the needle was gently inserted into the myocardium and slowly advanced towards the site of occlusion. The needle was visible through the LV free wall the entire time. When the needle reached the center of the LV wall, the plunger was gently pulled back to check for the presence of blood. If blood returns into the syringe, the needle is gently removed and reinserted at another site. If there is no sign of blood, than the cells or media are injected. Both the cells and the media caused a bleb to form on the surface of the LV. After injection, the needle was slowly withdrawn, and any apparent leakage or bleeding was noted.

At the end of the protocol the coronary artery in the OR group was re-occluded, and Unisperse Blue Dye (UBD, Ciba-Geigy, Hawthorne, New Jersey, 0.25 ml IV) was injected via the left jugular catheter in order to delineate the risk zone. While the animal was deeply anesthetized, potassium chloride (KCl, 0.5 ml IV, 2 mEq/ml) was then injected, to arrest the heart in diastole. The heart was excised, sliced transversely from apex to base into 4–5 slices, and photographed for calculating the area at risk (AR). The slices were then placed in triphenyltetrazolium chloride (TTC) for 10 min before being photographed again for delineation of the area of necrosis (AN). The slices were weighed, placed in formalin and submitted for histological analysis. By projecting the photographs of the hearts pre-TTC, we created tracings of each slice consist-
ing of any cavity, the area free of UBD, and the tissue containing UBD. Post-TTC tracings had cavity measurements (if present), white areas of necrotic tissue, and red stained normal myocardium. Both sets of tracings were then manually digitized (Sigma Scan) and the resulting measurements were used to calculate AR and AN as a percentage of the left ventricle. Infarct size is expressed as the percentage of the AN within the AR.

Hearts submitted for histology were embedded in paraffin, serially sectioned at 5 μm, and stained with hematoxylin and eosin and smooth muscle alpha actin, as a marker for embryonic cardiomyocytes [11]. Quantification of the risk zone that still contained the transplanted neonatal cells was done by using a 10× objective lens and a 10× eyepiece with a grid containing 100 squares (10×10 squares). Each square on the grid was considered as a field, and results were presented as a number of fields containing transplanted cells. For each heart, all slices containing any risk area (area within the LV devoid of blue-stained capillaries) were assessed. Neonatal cells are round and have a large nucleus that stains blue, making them easy to distinguish from the pink-stained cytoplasm of the native adult myocardial tissue (Fig. 1). Squares were counted positive if they contained neonatal cells and negative if they did not. On average 790 squares (fields) were counted per heart.

1.4. PCR analysis

Transplanted neonatal cells represented a mixture of both male and female cells. Since we used female rats as recipient animals, it was possible to track the implanted cells by determining the distribution of Sry gene, a marker of Y-chromosome present only in male cells. Originally, the liver, lungs, kidneys, and spleen were also excised, frozen in liquid nitrogen and stored at −80 °C for PCR analysis. However, we observed that the UBD interfered with the PCR reaction when analysis of the organs from the histology group was performed. In order to perform PCR-based DNA amplification, the rats (n=12) used for this analysis did not receive blue dye. Accordingly, for the PCR analysis we utilized five rats in each of the cell injection groups; two additional rats receiving media-injection (no cells), one in the OR and one in the PO group as negative controls.

Sry DNA was amplified by using polymerase chain reaction. At the end of the protocol after animals were given KCl, the heart, liver, lungs, kidneys and spleen were immediately excised and placed in liquid nitrogen, and stored at −80 °C until analyzed. Frozen tissues were weighed and minced with scissors in a digestion buffer containing 100 mM Tris–HCl pH8.5, 5 mM EDTA, 200 mM NaCl and 0.25% SDS (2 ml buffer/100 mg of tissue). The entire heart, lungs, kidneys and spleen were digested. Each liver was sampled three times, and these tissue aliquots were digested. Digestion was carried out at 55 °C for 18 h in the presence of protease K (final concentration 250 μg/ml). Genomic DNA from digested tissues was extracted with phenol: chloroform: isoamyl alcohol (25:24:1), precipitated with absolute ethanol, and washed two times with 75% ethanol. Washed DNA was dissolved in Tris–EDTA buffer (pH 8.0). PCR for the Sry gene was carried out with 35 cycles (each included 1 min at 94 °C, 1 min at 70 °C and 1 min at 72 °C) in the presence of all four deoxynucleotide triphosphates (final concentration 0.2 mM), MgCl2 (1.5 mM), 1U of Taq polymerase, and 25 pmol of each primer. The forward primer was represented by AGT GTT CAG CCC TAC AGC CTG AGG AC oligomer, and the reversed one—by GTG TGT AGG TTG TTG TCC CAT TGC AGC. Template DNA from each tissue sample was studied in two concentrations (0.5 and 1.0 μg/PCR tube). The size of the PCR product was 411 base pairs [10].

1.5. Statistical analysis

SAS software (SAS Institute, Cary, North Carolina) was used for statistical data analysis. Hemodynamic data was analyzed by using two-way ANOVA for repeated measures (univariate mode) followed by contrast transformation. Infarct size and histology data were compared by using t-test. Data are presented as mean±S.E.M. Values were considered to differ at a value of p<0.05.

2. Results

Twenty-five (OR group) and twenty-three (PO group) animals survived in each of the cell treated groups; five from each group were used for PCR analysis. Hemody-
Data presented as mean ± S.E.M.

Differences between the groups are non-significant.

* 239 min of occlusion in permanent occlusion group.

Fig. 2. Neonatal cardiac cells stained positive for the presence of the smooth muscle alpha actin isomorf (an embryonic marker in cardiac cells) are round in shape, and dark brown in color. In contrast to the host myocardium, which stains a pale brown or tan. Blood vessels, that contain smooth muscle cells, also stain positive for smooth muscle alpha actin. Original magnification 200× (10× eyepiece and 20× lens).

Fig. 3. Number of fields within the risk zone containing neonatal cardiomyocytes. Each field is represented by a square on the grid located on the eye piece. On average 790 fields were counted per each heart. *p < 0.05 vs. the permanently occluded group.

The PO group and 432 ± 84 cardiomyocytes in the OR group respectively (p = 0.12). Thus both counting methods; i.e. counting of cell positive fields per slide and the number of cells present in these fields indicated that there was a tendency towards decreased amount of cells in the OR group. In addition to cells present in the myocardium per se, we observed immature cardiac cells within the vasculature of the heart in both the PO and the OR groups as well (Fig. 4A–C).

In the OR group, all five rats (100%) demonstrated the presence of Sry DNA in the hearts and in the lungs. In two rats (40%), the Sry gene was found in both the liver and kidneys, and there was no detectable signal present in the spleen from any of the animals in this group (Fig. 5). In the PO group, the presence of the Sry gene DNA was documented in the hearts and lungs of all five rats (100%). Three rats (60%) had strong Sry signal in the kidneys, and a weak signal in the liver, and two rats (40%) demonstrated Sry presence in the spleen (Fig. 6). When both
media-injected rats were analyzed, no PCR signal was found in any of the organs studied.

3. Discussion

The major finding of this study supports the hypothesis [2,10] that injected neonatal cardiomyocytes are flushed from the site of injection. The transplanted neonatal cells are then redistributed to the other organs, primarily the lungs, but also to the liver, kidneys, and spleen, despite the fact that the cells were injected under direct visualization into the wall of the left ventricle. Reperfusion was associated with greater washout from the heart than occurred in the permanently occluded group. This is likely due to greater blood flow out of the previously ischemic area. The fact that the neonatal cardiomyocytes could often be observed within the cardiac vascular system suggests that this is the most likely route of washout.

Injecting cells of various lineages is a feasible option for repairing damaged myocardium, and improving cardiac function [2,12–14]. However, cell loss from the site of transplantation might be a problem and studying its mechanism(s) could help us to improve the outcomes of cellular cardiomyoplasty.

When cell loss after transplantation was first reported, several mechanisms including physical stress during injection, inflammation, rejection, hypoxic or apoptotic death were attributed as the cause. We postulated that the AR in the PO group would contain more neonatal cells than the OR group because of the lack of reperfusion. Only 4 of 20 rats (one-fifth) in the OR group had greater than 5% of the risk area occupied by the transplanted cells. In contrast, the

![Fig. 4. A. Two separate blood vessels that contain both red blood cells and round immature cardiac cells (original magnification 200×; i.e. 10× eyepiece and 20× lens). B and C are high power (original magnification 400×; i.e. 10× eyepiece and 40× lens) views of the two vessels from A. The neonatal cells are easily identifiable by their round shape and large nucleus (arrows). The vessels contain endothelial lining, and endothelial cell nuclei.](image)

![Fig. 5. Occlusion/reperfusion group. PCR product for the Sry gene amplified from DNA extracted from female rats receiving intramyocardial transplantation of both male and female neonatal cardiomyocytes. Note the signals for the Sry gene in the heart and lungs of all five rats, and signals in the liver and kidneys of rats 9338 and 9344.](image)
PO group had nine of 18 animals (one-half) with a greater than 5% occupancy of neonatal cells within the AR. This difference reinforces our belief that "washout" is detrimental to cell transplantation within the myocardium. The magnitude of cell washout may also depend on the presence of cell trafficking and/or homing factors in transplanted cells. The lack of these factors in transplanted cells could enhance the passive washout, while their presence might counteract the passive escape of transplanted cells. To address this issue we examined the expression of SDF 1/CXCR 4 axis in the neonatal rat cardiomyocytes. Isolated cells were subjected to immunohistochemical staining by using rat specific antibodies for SDF 1-alpha and CXCR 4 (Torrey Pines Biolabs, Inc., Houston, Texas). We observed positive staining for both factors (i.e. SDF1-alpha and CXCR4). These data suggest that interaction via SDF 1-alpha and CXCR 4 could be a factor, supporting the homing of transplanted cardiomyocytes and counteracting their passive escape.

Among other factors that could contribute to the cell loss from the myocardium are apoptosis and death due to trauma from the injection process. Müller-Ehmsen [10] looked at cell survival and reported that 1 h post injection into nonischemic hearts only 57% of the cells could be found by quantitative TaqMan PCR. This drop continued for at least 12 weeks at which time only about 15% of the cells could be accounted for. The caspase inhibitor AcYVADcmk (an anti-apoptotic drug) failed to improve the number of surviving cells. Based on these results one might suggest that caspase-dependent apoptotic cell death is not taking place in the transplanted neonatal cardiomyocytes. However, Müller-Ehmsen’s study was performed in non-infarcted hearts. In our study, we injected neonatal cells into the ischemic myocardium, where apoptosis could be a factor. In the Müller-Ehmsen’s study [10] only the caspase-specific apoptotic pathway had been targeted by this broad-spectrum caspase inhibitor. Thus, the involvement of other apoptotic pathways (e.g. caspase-independent DNA degradation by apoptosis-inducing factor) could be a factor. Considering these facts, the possibility of apoptosis-related processes in the transplanted neonatal cardiomyocytes cannot be ruled out. Neither inflammation nor rejection (Fischer rats are syngeneic) was observed histologically during the course of this study; and it is unlikely that rejection would have occurred within 3 h of injection. Other factors that might contribute to the loss of transplanted cells are generation of oxygen reactive species and release of toxic cytokines. All the above mentioned factors (i.e. apoptosis, reactive oxygen species and cytokines) are known to contribute to the cell death in the ischemic myocardium, and could be involved in the death of transplanted neonatal cells as well. In addition to these already known mechanisms that might cause a decrease in the number of transplanted cells, we now describe a new one; i.e. the cell escape phenomenon by which the transplanted cells are removed from the site of transplantation.

By utilizing the DNA marker for the Sry gene in the transplanted male cells, we could determine where the cells were going. Even though this technique does not quantify the number of cells present in the remote organs, it does show to which organs the cells travel. PCR analysis revealed that the lungs had the strongest and most consistent Sry signal compared to the other organs studied. The signals for the Sry gene in the heart and lungs of all five rats, signals in the liver and kidneys of rats 9309, 9339 and 9343, as well as the spleens of rats 9244 and 9339.

Fig. 6. Permanently occluded group. PCR product for the Sry gene amplified from DNA extracted from female rats receiving intramyocardial transplantation of both male and female neonatal cardiomyocytes. Note the signals for the Sry gene in the heart and lungs of all five rats, signals in the liver and kidneys of rats 9309, 9339 and 9343, as well as the spleens of rats 9244 and 9339.
Intracoronary infusion of bone marrow- or circulating blood-derived progenitor cells to patients with acute myocardial infarction resulted in a significant increase in global left ventricular ejection fraction, improved regional wall motion in the infarcted area, and reduced end-systolic left ventricular volumes at 4 month follow-up [9,15]. These clinical data undoubtedly indicate that cell transplantation improves functional performance in damaged heart. However, the cellular mechanism of this beneficial effect has yet to be determined. The fate of transplanted cells is an important issue. The current study indicates that transplanted cells can escape from the heart and travel to distant organs. To our knowledge this is the first time that the phenomenon of cell escape through the cardiac vasculature has been reported. One limitation of this study is that we did not quantitate the degree of washout to other organs. However, our basic goals were to determine whether the cells escape the site of transplantation, how they escape, and if coronary blood flow would effect the distribution of transplanted cells. In addition, we consider the use of neonatal cells as an experimental approach aimed at characterizing the fate of transplanted cells (i.e. any cells), and developing basic concepts for cell transplantation.

Cell escape, or wash out, from the site of transplantation is a factor that could contribute considerably to cell loss. This phenomenon is more pronounced when the coronary artery is reperfused compared to an artery that is permanently occluded. Improvements in transplantation techniques and the development of new approaches aimed at reducing or preventing cell escape could greatly contribute to the future success of the field of cellular transplantation.

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References


