

Engraftment of pre-differentiated stem cells into cardiomyocytes in an animal model of ischemic cardiopathy

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ABSTRACT

Stem cell therapy for cardiac infarct regeneration has been widely used in clinical research. Despite the fact that important advances in this field have been reached, the observed recovery does not demonstrate new cardiac muscle formation. Benefits have been observed due to an improvement in neovascularisation. The main objective of this study was to determine if pre-differentiated stem cells into cells of myocardic lineage are capable of engraftment in animal models with induced cardiac infarct and are capable of truly differentiating into cardiomyocytes. Bone marrow rat stem cells were pre-differentiated with 5-AZ. After 4 weeks, pre-differentiated stem cells express muscarinic 1, 2 and β adrenergic 2 receptors. Also, proteins such as sarcomeric α -actin, cardiac myosin heavy chain, desmin and vimentin were detected by immunocytochemistry. Cells were transplanted intracardially in an ischemic cardiac rat model. Pre-differentiated or non differentiated cells were transplanted after 4 weeks post infarct induction. Histopathology of the hearts was made 2 weeks after cell transplantation. Typical granulated tissue, scare formation and neovascularisation were observed in both groups. However, in those hearts from rats inoculated with pre-differentiated cells many appeared atypical and were α -actin sarcomeric positive. These events suggest that pre-differentiated cells conserve some muscle characteristic traits *in situ* that at least last for two weeks

after transplantation.

KEYWORDS

Cell Therapy; MSC; Regenerative Medicine; Cardiocyte Differentiation; Stem Cells

1. INTRODUCTION

Myocardial infarction is the leading cause of heart failure in developed countries. Even advanced therapeutic measures are usually not sufficient to prevent left ventricular remodeling as they fall short of replacing necrotic cardiac myocytes. Recent insights into stem cell biology have changed our understanding of regenerative activities in the infarcted heart and have raised considerable hopes for novel therapeutic approaches aimed at cardiac myocyte replacement/regeneration through cell transplantation.

Considering this fact, stems cells have been used for autologous transplant in several clinical trials for repair of the ischemic myocardium. The results show heart function improvement after stem cells transplantation [1-11]. However, It remains unclear which stem cell(s) can contribute to these effects on the myocardium, and whether they do so by trans-differentiation or cell fusion [12]. It has been suggested that this improvement of heart function has been the result of angiogenic effects more than the generation of new cardiac muscle [13].

Several reports of stem cells application into damage hearts have been made, but until now there is no a general consensus of what specific type of the generally named stem cells is the best option. Comparing the relative efficacies to facilitate recovery of cardiac damage,

different cells types have been studied: Bone marrow (BM) mononuclear cells showed to be more effective than BM mesenchymal stem cells (MSC) [14] and adult blood stem cells were more effective than cord blood stem cells [14]. Adipose derived stem cells (ASCs) have also been used for repair in myocardial infarction. Comparing the *in vivo* behavior of ASCs and MSCs in the necrotic heart demonstrated that, in general, both populations of stem cells do not tolerate the cardiac environment, resulting in acute donor cell death and a subsequent loss of cardiac function similar to control groups. Nevertheless, some tolerance, although not ideal, was observed with CD133⁺ MSCs [15]. According to Davy *et al.* [16] enrichment of autologous bone marrow CD34⁺ stem cells could increase the efficacy of treatments in clinical settings. Despite the fact that CD34⁺ cells have been the selected option for heart repair, there are also some reports of CD133⁺ cells applied in clinical trials showing improvement of myocardial viability and local perfusion of the infarcted zone [17-22]. However, none of these studies have shown *de novo* formation of cardiac muscle.

On the other hand, it has demonstrated the capacity of adult stem cells for *in vitro* differentiation into cardiomyocytes. This possibility presents an alternative approach for transplant protocols of stem cells going into cardiomyocytes instead of using undifferentiated stem cells. 5-azacitidin (5-AZ) or its derivatives were reported to induce cardiac differentiation *in vitro* of mesenchymal stem cells derived from bone marrow and embryonic cells [23-27].

Hakuno report that differentiated mesenchymal cells derived from the bone marrow (CMGs) express β -adrenergic receptors as well as muscarinic M1 and M2 receptors [24]. Furthermore, stimulation of CMG cells with phenylephrine, isoproterenol, or carbachol could activate downstream signalling pathways through specific receptors. Temporal expression of β 1- and β 2-adrenergic and M1 and M2 muscarinic receptors was reported [23]. The main objective of this study was to determine if pre-differentiated stem cells into myocardial lineage cells are capable of engraftment in animal models with induced cardiac infarct and truly differentiate in cardiomyocytes, instead of endothelial cells.

2. MATERIALS AND METHODS

2.1. Isolation, Expansion, and Myocardial Induction of Rat Bone Marrow-Derived Stem Cells

Female Wistar rats were anesthetized with ether, thigh bones were excised, and bone marrow cells were obtained. Cells were cultured for 24 hs on 60-mm dishes in Dulbecco's Modification of Eagle's medium (DMEM-

F12) (GIBCO BRL) supplemented with 20% fetal bovine sera (FBS) and penicillin (100 μ g/ml)/ streptomycin (250 ng/ml) at 37°C in humid air with 5% CO₂. After that the media were change and maintained in DMEM-F12 medium supplemented with 10% FBS and antibiotics for several weeks.

2.2. Stem Cell Culture and 5-AZ Treatment

To induce cell differentiation, cells from the third passage were incubated in DMEM-F12 medium supplemented with 5% FBS and antibiotics 24 h before treatment with 3 μ mol/l of 5-azacytidine (Sigma Chemical Co., St. Louis, Missouri, USA) for 24 hours. After incubation, media were change and maintained on DMEM-F12 medium supplemented with 10% FBS and antibiotics for four weeks. In week 4, these cells were used for RT-PCR expression of muscarinic and adrenergic receptor, immunodetection of myosin, actin, desmin and vimentin, and animal transplantation.

2.3. Reverse Transcription-Polymerase Chain for Myocardial Receptors

Total RNA from 1 to 10 weeks after 5-AZ induction cells was extracted with GenElute mammalian total RNA (Sigma Chemical Co., St. Louis, Missouri, USA). Reverse transcription-polymerase chain reaction (RT-PCR) was performed with OneStep RT-PCR Kit (Qiagen). Amplification was performed for genes responsible for the expressions of β 1A, β 1B, M1, and M2 receptors. The primers used are listed in **Table 1**. HRTF was used as an internal control for each sample.

2.4. Immunocytochemistry for Myocardial Proteins

Cardiomyocytes protein synthesis was analyzed by immunostaining with monoclonal antibody (MF20) to sarcomeric α -actin, cardiac myosin heavy chain, desmin,

Table 1. Primers used for gene expression.

Gene	Primer sequence (5'-3') forward
β 1 (Forward)	5'ACGCTCACCAACCTCTTCAT3'
(Reverse)	5'AGGGGCACGTAGAAGGAGAC3'
β 2 (Forward)	5'CCTCATGTCCGTTATCGTCC3'
(Reverse)	5'GGCACGTAGAAAGACACAATC3'
M1 (Forward)	5'CTGGTTTCCTTCGTTCTCTG3'
(Reverse)	5'GCTGCCTTCTCTCCTTGAC3'
M2 (Forward)	5'GGCAAGCAAGAGTAGAATAAA3'
(Reverse)	5'GCCAACAGGATAGCCAAGATT3'

and vimentin (Sigma Chemical Co., St. Louis, Missouri, USA). Cells grown on glass coverslips were fixed with -20°C methanol and permeabilized with 0.3% Tritón X-100 (Sigma Chemical Co., St. Louis, Missouri, USA) in PBS. After blocking with 5% BSA in PBS for 1 h at room temperature, the cells were incubated with different fluorescent primary antibodies. Fluorescein/conjugated anti-mouse IgG (Pierce) were used as second antibody. Visualization was performed on epifluorescence AXIO IMAGER.Z1 microscope.

2.5. Myocardial Infarction Induced in Wistar Rat

Myocardial infarction was performed under anesthesia with an intraperitoneal injection of pentobarbital 40 mg/kg. Trichotomy was undertaken in the neck region. The neck dissection was carried out by planes into the trachea to culminate with a tracheostomy. A tracheal tube was placed (Jelco No. 16 Braun Introcán[®]). Ventilation frequency and tidal volume for mechanically ventilated was calculated by positive pressure (Kent Scientific Dual Mode TopoM). Midline sternum incision was performed and dissected at all tissue levels to the bone. With slight pressure on the heart with an applicator or spatula, heart rate was decreased. Then we proceeded to perform a surgical obstruction midway of the left anterior descending coronary artery with 6-0 Prolene suture. At the end, closure of the rib cage in layers with Vicryl suture 5-0 was done.

2.6. Stem Cells Transplantation into Rat Infarct Heart

After 4 weeks of myocardial infarction, three groups consisting of six male Wistar rats each were established. Group I: Intracardiac injection at the site of infarct with PBS. Group II: Intracardiac injection at the site of infarct with non-differentiated stem cells. Group III: Intracardiac injection at the site of infarct with stem cells incubated in induction medium for 4 weeks (pre-differentiated to cardiocytes). The heart was exposed as previously described and $1 \times 10^5/100 \mu\text{l}$ of non differentiated or pre-differentiated stem cells were injected in the infarcted area and proceeded to close as previous.

2.7. Histological Analysis of Transplanted Rat Heart

Four week after cells transplantation or PBS injection animals were sacrificed and hearts fixated with a solution of 10% PBS/formaldehyde and embedded in paraffin. Serial sections were performed at the infarcted area. Lamella preparation was stained with hematoxylin/eosin and Masson trichrome stain for interpretation and evaluation of parameters of healing of the infarcted area.

3. RESULTS

3.1. Cell Culture and 5-AZ Treatment

Primary culture of stem cells recovered from the rat bone marrow showed characteristic CPU formation after 12 days incubation in DMEM-F12 medium (**Figure 1(a)**).

After 4 weeks of 5-AZ treatment the percentage of cardiomyocytes-like cells was approximately 30% to 50%. In addition, there were important morphology changes on 5-AZ treated cells cultured after 3 weeks, containing large structures different to the ones observed in the colony formation units (CFU) or with the untreated cultures (**Figure 1(b)**).

3.2. Immunodetection

Four weeks after culture exposure to 5-AZA, the cells showed positive staining for vimentin (**Figure 2(a)**), cardiac myosin (**Figure 2(b)**), sarcomeric α -actin (**Figure 2(c)**) and desmin (**Figure 2(d)**) by immunohisto-

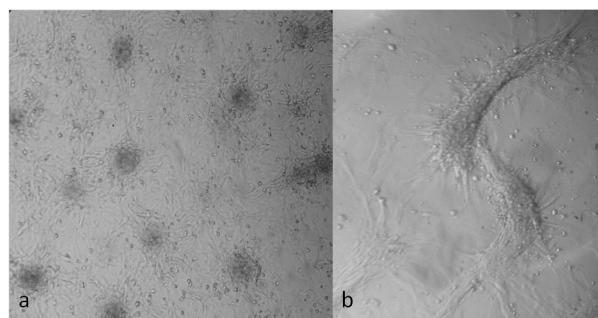


Figure 1. Primary cultures of rat bone marrow stem cells before and after 5-AZ incubation: (a) Several CFUs on a confluent plate after incubation for 12 days in DMEM-F12 medium; (b) Morphological and disposition changes of organized cells on a plate, after 4 weeks of 5-AZ induction.

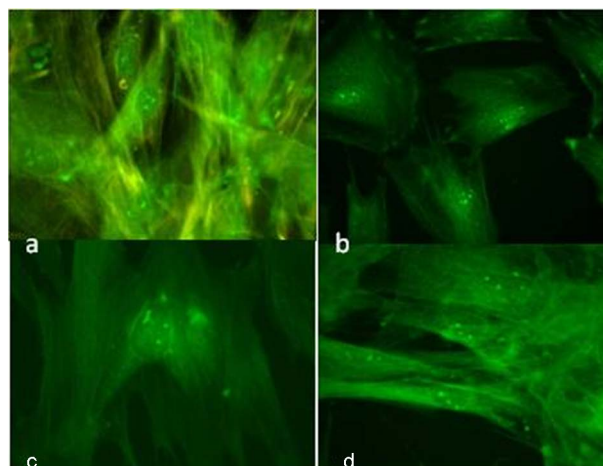


Figure 2. Microphotography of bone marrow stem cells in culture after 4 weeks of 5-AZ induction. Positive immunostaining of cardiac proteins. Vimentin (a), cardiac myosin (b), α -actin sarcomeric (c) and desmin (d) 100 \times .

chemistry (Figure 2).

3.3. Reverse Transcription-Polymerase Chain

RT-PCR showed the presence of muscarinic-2 receptor expression on differentiated stem cells that increased 4 weeks after 5-AZ treatment. Muscarinic -1 and 2 receptors were detected weakly until 4 weeks as well as the Adrenergic β_2 receptor. Adrenergic β_1 receptor was un-detected in any sample (Figure 3).

3.4. Macroscopic and Microscopic Analysis of Myocardial Infarct Area after 2 Weeks of Stem Cells Transplantation

All experimental groups showed macro-and microscopic evidence of acute myocardial infarction. In the hearts from control animals, a macroscopic area of gray-whiteish scar progressing from the edge to the center of the infarct around the suture was observed (Figure 4(a)). This was less evident in hearts transplanted with predifferentiated stem cells (Figure 4(b)) or stem cells without differentiation (Figure 4(c)).

The histopathological analysis of the lesion confirmed the presence of a variable area of granulated tissue associated with replacement at different stages of fibrosis or scar tissue with abundant collagen deposition (Figures 5(a), (b)). The histopathological analysis of heart from animals which were transplanted with stem cells without

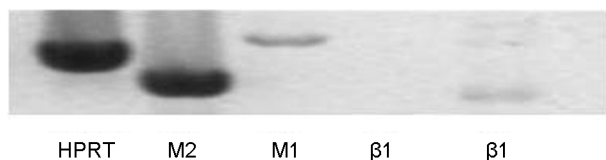


Figure 3. Detection by RT-PCR of the expression of Muscarinic 1 (M1), Muscarinic 2 (M2) and Adrenergic β_2 receptors on bone marrow stem cells after 4 week of 5-AZ induction.

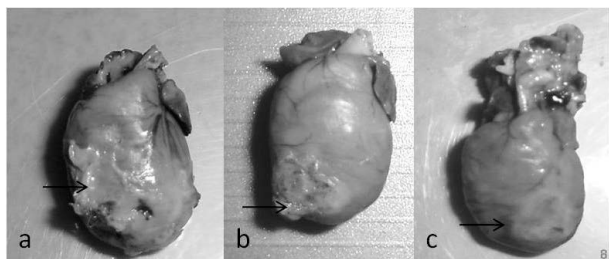


Figure 4. Macroscopic images of hearts from infarcted rats. (a) The Heart from a rat of the control group showing an extended area of damage in the infarcted area; (b). Heart from a rat inoculated with 10^5 of non- differentiated stem cells. It demonstrates a less extended area of damage in the infarcted area; (c) Heart from a rat inoculated with 10^5 of pre-differentiated stem cell. It demonstrates an apparent lesser damage in the infarcted area.

differentiation show the presence of a variable area of granulation tissue associated with replacement at different stages of fibrosis or scar tissue with collagen deposition and vascular congestion (Figures 5(c), (d)). Hearts from rats transplanted with stem cells pre-differentiated to myocytes present also a larger area of granulated tissue or neovascularization, however there was a significant presence of reactive fibroblasts and atypical cells (arrow) (Figures 5(e), (f)). Also the immunohistological analysis demonstrated that the atypical cells, than do not resemble cardiac muscle fiber, were positive for sarcomeric actin (Figure 6).

4. DISCUSSION

It has been proposed that the benefit of stem cell transplantation on a myocardial infarct is owed to neo-vascularization, because these cells tend to differentiate into endothelial cells instead of cardiocytes. The aim of this work was to analyze the possibility of true myocardial cell engraftment if pre-differentiated cardiocytes, or undifferentiated stem cells were used. The pluripotency and plasticity of stem cells had been de-

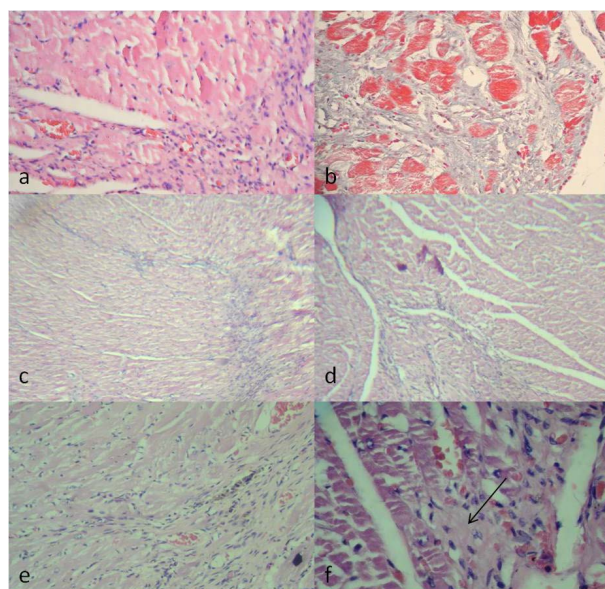


Figure 5. Light micrographs of transversal ventricular sections. (a) Rat infarct heart 2 weeks after injecting PBS. The Hematoxylin & Eosin staining of this heart exhibits increased interstitial fibrosis, granulation and vascular congestion; (b) Rat infarct heart 2 weeks after injecting PBS. Masson Trichromic staining exhibit high collagen content; (c) Rat infarct heart 2 weeks after the injection of undifferentiated bone marrow stem cells. Hematoxylin & Eosin staining of this heart exhibit granulation; (d) Rat infarct heart 2 weeks after injecting undifferentiated bone marrow stem cells. Masson Trichromic staining of this heart exhibit collagen deposit; (e) Rat infarct heart 2 weeks after injecting predifferentiated bone marrow stem cells. Hematoxylin & Eosin staining of this heart exhibit granulation; (f) atypical cells.

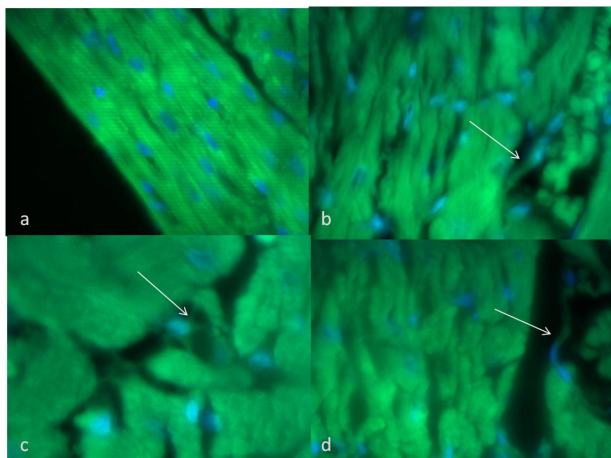


Figure 6. Immunohistological detection of sarcomeric actin (a) Positive immunostaining against α -actin sarcomeric in normal cardiac tissue; (b)-(c) Histological view of experimental heart after pre-induced miocardial cells transplantation showing altered histology area and atypical cells (arrow).

monstrated. Also it has been shown that depending on the signals in the environment the cells can differentiate into specific cell lineages [26-30]. Reports of stem cells transplanted into animal models which described an increase in vascular tissue, but not of cardiac muscle mass are available [13]. This could be a consequence of the presence of inflammatory macrophages at the niche or implantation site. By inducing the formation of new vessels due to the activation of angiogenic signals by these cells, the transformation to endothelial cells instead of cardiocytes could be explained. However, if genes are switched on with anticipation into a cardiac lineage, the probability that these cells complete their differentiation into cardiomyocytes by transdifferentiation *in situ*, opens an interesting possibility for the true replacement of lost tissue. In fact, there is a report of autologous cardiac stem cells transplantation in patients with heart failure of ischemic etiology, induce striking improvement in both global and regional left ventricular function, a reduction in infarct size, and an increase in viable tissue. However the isolation of the cardiac stem cells must be obtained during coronary artery bypass grafting [31-33]. However, the application of cardiomyocytes precursors, could be an alternative. The results of this study demonstrate that stem cells stimulated with 5-AZ are capable of expressing specific cardiac protein such as actin, myosin, desmin and vimentin, as well as the expression of adrenergic and muscarinic receptors, but with no full differentiation into cardiomyocytes. The latter concept is important because the pre-differentiation could allow these cells to engraft correctly and contribute to an increase in cardiac muscle mass. The presence of atypical cells in the hearts of transplanted animals should be followed up more time in order to verify whether or not these atypical cells could transdifferentiate into new muscle tissue and

not only in scar or vascular tissue.

5. CONCLUSION

Detection of atypical cells α -actin sarcomeric positive on cardiac damage tissue after two weeks of animals transplanted with pre-differentiated cells suggests that these cells could be a better option for heart repair.

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