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Mesenchymal stem cell administration at coronary artery reperfusion in the rat by two delivery routes: a quantitative assessment

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Abstract

Aims—Ideally, mesenchymal stem cells (MSC) home to and/or remain at the site of damaged myocardium when administered after myocardial infarction. However, MSC may not remain in the heart, but instead relocate to other areas. We investigated quantitatively the distribution of labeled rat MSC, given by two routes after coronary artery occlusion/reperfusion in rats.

Main Methods—Rats were subjected to 45 minutes of coronary artery occlusion and 7 days of reperfusion. Before reperfusion rats received 2×10^6 MSC, labeled with europium, injected directly into the ischemic region of the heart ($n=9$) or intravenously ($n=8$). After one week tissues were analyzed for label content together with a standard curve of known quantities of labeled MSC.

Key Findings—In rats receiving cells injected directly into the myocardium, 15% of labeled cells were retained in the heart. When the cells were administered intravenously, no MSC were detected in the heart. The route of administration did not affect distribution to other organs, as the number of MSC in liver, spleen and lung was similar with both routes of delivery.

Significance—Even with direct intramyocardial injection, only a small proportion of the cells are retained in the heart, instead traveling to other organs. With intravenous injection there was no evidence that cells "homed" to the damaged heart. Although cell delivery to the heart was significantly affected by the route of administration, the distribution of cells to other organs was similar with both routes of administration.

Keywords

cell therapy; infarction; reperfusion; rat mesenchymal stem cell; stem cell administration; nanoparticle; neutron activation

Introduction

Stem cell therapy for the treatment of myocardial infarction has gained a wide acceptance in the medical community, and human clinical trials are being performed in an attempt to regenerate heart tissue and to improve function in the infarcted heart. These early clinical

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studies have shown only modest effect on cardiac function, negative effect or transient effect only, indicating that despite the promise of cell therapy, factors such as cell type, dose (number of injected cells) and the precise timing and route of the injection still need to be determined.

Although many types of cells have been tested, including unpurified bone marrow (Orlic et al. 2001), neonatal cardiomyocytes (Muller-Ehmsen et al. 2002), fetal cardiomyocytes (Yao et al. 2003), skeletal myoblasts (Menache, 2004) and endothelial progenitor cells (Kawamoto et al. 2001), mesenchymal stem cells (MSC) show promise since they can be expanded in large numbers, can be used allogeneically without immune suppression, can express cardiac markers, and have been shown to improve cardiac function, possibly through a paracrine effect. Whether MSC truly differentiate into adult-type cardiomyocytes remains controversial (Makino et al. 1999; Toma et al. 2002; Murry et al. 2004; Dai et al. 2005).

Some studies have shown that MSC may have the ability to home to the site of injury when administered intravenously after myocardial infarction (Price et al. 2006; Krause et al. 2007; Nagaya et al. 2004; Barbash et al. 2003). This route of delivery is appealing since it is not invasive, large numbers of cells can be administered, and it has been shown to be safe in human trials (Hare et al. 2007). However, potential disadvantages of systemic delivery can be low uptake/retention in the infarcted heart and cell relocation to organs other than the heart. This relocation phenomenon is a problem even when cells are injected directly into the muscle or scar of the heart, as cells may move out of the injected area and relocate to other sites in the body (Dow et al. 2005).

There are ~ 40 million cardiomyocytes in one gram of normal rat heart tissue (Van der Laarse et al. 1987). In a rat heart that weighs 1 g, an infarct comprising 20% of the left ventricle would require about 8 million cells for one-to-one replacement of dead myocytes; the human hearts average 200 to 300 grams, so if direct replacement of dead myocytes were required to restore normal geometry of the heart, a very large dose of cells would be required. However many preclinical studies suggest that only a small number of stem cells may provide a functional benefit and enhance angiogenesis (Gnecchi et al. 2006; Dai et al. 2007), although the mechanisms that have yet to be determined. In addition, the proliferative nature of immature cells may allow fewer to be injected.

Both the timing and route of administration will be important variables for successful application of cell therapy. Early in the evolving infarct the inflammatory process begins and cytokines that may be related to the homing process of stem cells are released. The appropriate window of treatment for either reducing infarct size or improving remodeling is unknown at this time.

The purpose of this study was to determine whether MSC can survive and target the myocardium when transplanted immediately after myocardial infarction in a model of coronary artery occlusion followed by reperfusion. In addition, we tested whether the route of delivery, i.e. intravenous administration or direct intramyocardial injection alters localization in the jeopardized region of the heart. To establish cell distribution, we labeled the MSC with nanoparticles containing europium. The advantages of this technique are that 1) the label is non-radioactive (does not expose either cells or recipient to radiation); 2) the nanoparticles are taken up and entrapped within cytoplasmic vesicles and therefore do not readily diffuse from the cell, and 3) unlike fluorescent labeling, this technique is quantitative. Because neutrons are not attenuated in a sample, measurements can be made in tissues without the need for extraction (Vaccaro et al. 2006). This technique of neutron activation, using the isotope iridium, has been used previously to track cells in a pig model (Freyman et al. 2006). Nanoparticles have been used for many years to track labeled cells using Magnetic Resonance Imaging (for example

see Hill et al. 2003). Although the MRI technique can localize implanted cells, it does not allow quantification of the cells.

Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee of Good Samaritan Hospital, and the investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Cell Labeling and Counting

Adult bone marrow-derived stem cells derived from Lewis rats, at passage 6, were obtained from the Tulane University stem cell bank. The cells were expanded in culture in complete culture medium (CCM) containing α - Minimal Essential Medium Eagle with L-glutamate and without deoxyribonucleosides (400 ml), fetal bovine serum (100 ml), antibiotic/antimycotic (5 ml), and L-glutamate (10 ml). On the last day of expansion the cells were labeled with the europium-containing nanoparticles (Biophysics Assay Laboratory, Worcester, MA) at a 1:100 concentration in medium for 21 hours. This concentration and timing was shown to be suitable for our cells by a previous pilot study performed in our laboratory. The cells were then washed, placed in tubes with medium composed of CCM + 5% DMSO. The tubes were put in a cryo-container containing isopropanol and frozen overnight in a -70°C freezer. The tubes were then transferred to storage in liquid nitrogen until use. Two expansions were used in this study.

The tracer used in this study was composed of a colloidal material in which a lanthanide metal, in this case europium, was embedded in a nanoparticle of approximately 20 nm (BioPAL, Biophysics Laboratory, Worcester, MA). The cells were incubated in medium containing the tracer. The nanoparticles containing the europium have been shown to incorporate into cytoplasmic vacuoles within the cells (Vaccaro et al. 2006). A standard curve of various cell numbers was prepared. Cells were administered to the rats as described below. At the end of the study, tissue samples were obtained from the animal and desiccated. The samples were sent to Biophysics Laboratory and were subjected to neutron activation. The assay for europium was performed by exposing the samples containing the nanoparticles to a neutron beam. The neutrons combine with the atoms of the metallic element of the probe causing these atoms to become radioactive. These unstable atoms release energy in several forms including high-energy photons which can be quantified in an automatic spectroscopic instrument. The disintegrations per minute (dpm) were reported for each sample, and the number of cells present in the tissue was then calculated using the standard curve.

Surgical Preparation and Cell Injection

Adult Fischer female rats (130–180 g) were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (5 mg/kg), intubated, and ventilated with room air. The chest was shaved and swabbed with betadine and then alcohol. The chest wall was infiltrated with bupivacaine (0.1 mg/kg). The thorax was opened under sterile procedure (at the fourth intercostal space) and the pericardium excised. A stitch was taken around the left coronary artery (4-0 suture with an atraumatic needle) entering the myocardium below the atrio-ventricular groove and exiting close to the pulmonary cone just beneath the lower edge of the left atrial appendage. The ends of the suture were threaded through a piece of plastic tubing forming a snare that could be used to occlude and reperfuse the artery. The rats were subjected to 45 minutes of coronary artery occlusion, and 5 minutes before reperfusion, rats randomly received 2×10^6 labeled MSC, injected either directly into the ischemic anterior free wall of the heart using a 0.5 ml insulin syringe with attached 28-gauge, 1/2 inch needle bent at a 45 degree angle (70 μl , n = 9) or through a PE-50 catheter placed in the jugular vein (1 ml, n =

8). Incisions in the neck and chest were closed, buprenorphine (0.02 mg/kg subcutaneously) and saline 10 ml/kg (subcutaneously) were administered, and after weaning from the respirator, the rats were placed on a heating pad while recovering from anesthesia. After 1 week, the rats were reanesthetized and euthanized, and tissues were obtained from heart, lung, kidney, spleen, and liver. In the heart, the right ventricle was removed and the left ventricle was cut into two sections: 1. the anterior free wall (site of infarction) and 2. posterior wall plus septum (remote from the site of infarction). Samples were taken from lung, kidney, spleen and liver and were weighed. All samples were then desiccated and sent for counting.

Statistical analysis

Wilcoxon's non-parametric statistic was used to compare the distribution by the two routes of administration to lung, kidney, liver and spleen.

Results

Relationship between europium content and cell number

Standard curves of known quantities of labeled cells were prepared in vitro and analyzed by the neutron activation method. Figure 1 shows the relationship between the number of cells and the disintegrations per minute for each sample in one expansion. The variables were linearly related and showed a good correlation in both expansions ($r = 0.99$). The limit of detection was 10,000 cells.

Animals and treatment

Nineteen rats were assigned to the study. One rat died during coronary artery occlusion prior to cell injection and one rat (assigned to direct injection) died at 10' after reperfusion. Data are reported in 9 hearts in the direct intracardiac injection group and 8 hearts in the intravenous administration group.

Cell Retention and Relocation

Table 1 shows data from the hearts of individual rats in both groups. In rats receiving MSC injected directly into the heart just before reperfusion, on average 299,041 cells or 15% of the labeled cells remained in the left ventricle (infarcted and non-infarcted areas) after 1 week. Cells were present in both non-infarcted and infarcted regions of the heart, with the bulk of the cells present in the heart (82%) found in the infarcted portion. In rats receiving cells injected intravenously, no measurable cells were present in either the infarcted or non-infarcted regions of any heart.

Cells were also present in liver, lung and spleen of all rats and in the kidney of one rat. There were no significant differences in the accumulation of cells in these 3 organs when the cells were administered by direct intramuscular injection or by the intravenous route (Figure 2).

Discussion

For therapeutic application of stem cell therapy to be effective in treating myocardial infarction in humans, many factors still need to be resolved. One of these factors is the best method to use to deliver cells to infarcted myocardium. Table 2 summarizes techniques being used and their advantages and disadvantages. Also, passive or active migration of the injected stem cells to other parts of the body may become an important negative side effect of cell therapy, so it is important to determine the distribution of the cells after implantation. Although the intravenous approach is one of the least invasive, our results suggest that a direct intramuscular injection is more likely to lead to retention of cells in the area being treated than intravenous administration.

In the present study, we used a nanoparticle-uptake cell label to evaluate the distribution of cells in a quantitative fashion. Using this method, we could assess the presence of label in the entire heart, something not possible with more traditional methods utilizing genetic markers such as green fluorescent protein or dye labeling techniques such as DiI that depend on histologic assessment of cells in a relatively small percentage of the heart.

Data from the present study showed that we were able to detect the europium label in tissues and to determine the distribution in various organs. We observed that even when labeled cells were injected directly into the myocardium, the label was found in other organs days later. The number of labeled cells per gram detected in liver, spleen and lung was similar whether the cells were injected directly into the heart or given intravenously. With direct intramuscular injection more label was detected in the infarcted myocardium than in the non-infarcted regions of the heart. Direct intramuscular injection of the cells into the ischemic region resulted in some retention of cells in that area, whereas intravenous injection did not result in cell localization to the infarcted myocardium by homing.

Redistribution of cells after injection

When cells are injected directly into the myocardium, they may redistribute via several mechanisms. Cells can enter the myocardial circulation and be carried passively to other regions of the body. When cells are injected into the muscle just before coronary artery reperfusion, it is likely that reperfusion itself increases the amount of cells lost to the region. Dow and coworkers have shown that hearts with a reperfused coronary artery demonstrated cells in only about half as many microscopic fields of ischemic myocardium three hours later compared to hearts with permanent coronary artery occlusion. In that study, the injected cells could be visualized leaving the heart through venules (Dow et al. 2005). Data from the present investigation are consistent with that study: hearts receiving coronary artery reperfusion contained only 15% of labeled cells one week later, when the cells were injected directly into the heart. In addition, cells can be lost from the site of injection due to spilling from the needle hole.

In some hearts in the direct injection group there were cells present in the non-infarcted area while in others there were no cells present in this area. This may reflect movement of cells from the infarct into non-ischemic areas, or may be due to a technical problem with the injection and/or difficulty in identifying the appropriate areas when the tissue was sampled at the end of the protocol.

Detecting cells injected intravenously as potential therapy for myocardial infarction

Some studies have provided evidence that when administered systemically MSC target regions of tissue damage (so-called "homing"). Price et al. injected DiI labeled-MSC intravenously into pigs at 30 minutes after coronary artery occlusion and, in addition to the lungs, found labeled cells in the infarcted and peri-infarcted regions three months later. In cell-treated animals left ventricular function was better than in controls (Price et al, 2006). In a recent study by Krause and coworkers, MSC labeled with a fluorescent dye were located by histology in the peri-infarcted region of pig hearts at one month after being injected intravenously (Krause et al, 2007). They also observed infarct size reduction and an improvement in left ventricular function in the pigs that received MSC.

In the rat model of myocardial infarction, MSC injected intravenously after MI have been found in the heart hours to weeks later using techniques such as fluorescent labeling (Nagaya et al. 2004), BrdU labeling (Barbash et al. 2003; Ma et al. 2005) and by fluorescence in situ hybridization analysis for the Sry gene where male MSC were given to female hosts (Jiang et al. 2005). However in these studies only a small percentage of the total dose was observed in

the heart at a later time point. For example, Nagaya et al. using a semi-qualitative histologic analysis and fluorescent microscopy, estimated that only about 3% of the initial dose of 5 million cells was still present in the heart after 1 month (Nagaya et al. 2004). Barbash and coworkers found that within 4 hours after intravenous administration, fewer than 1% of technetium 99m-labeled cells administered intravenously were located in the heart as assessed by nuclear imaging (Barbash et al. 2003). As body mass increases, the vascular distance grows, making it more difficult for cells injected intravenously to "home" to the site of injury. Thus, the fact that small cell numbers may still have a beneficial impact is important. The presence of cells may impact angiogenesis or function even if they are not present in large enough numbers to replace muscle. For example, in experimental models some investigators have found improvement in function with cell implantation after MI even though cells are not present in large numbers. They attribute this functional improvement to some type of paracrine effect of the cells in the heart (Gnecchi et al. 2006; Dai et al. 2007).

Other studies are in agreement with our study and failed to locate MSC in the heart after intravenous administration. Chin et al. infused ¹¹¹Iridium oxine labeled MSC into pigs after MI and followed the distribution of the cells for 2 weeks using SPECT imaging. They found localization of cells in lungs, liver, spleen and bone marrow, but minimal accumulation in the heart (Chin et al. 2003). Freyman et al. tested 3 delivery routes for administration of MSC after MI in pigs. Nanoparticle-labeled cells (iridium) were given 15 minutes after myocardial infarction by direct injection into the heart, intracoronary injection or intravenously. At two weeks engrafted cells were found in the heart after direct injection and after intracoronary administration, but no cells were found in hearts of pigs who received MSC intravenously (Freyman et al. 2006). Taken together, data from these studies and ours may have important clinical significance, suggesting that intravenous administration of therapeutic cells may be a poor route of delivery in humans.

The disparity of the presence or absence of MSC located in the heart after intravenous administration in the above studies may be a question of sensitivity. The limit of detection with our technique was 10,000 cells. So although we detected no label in the heart with IV injection, it is possible that cells were present in small numbers. Even a small number of engrafted MSC might be enough to benefit the heart due to indirect effects of cell engraftment. Nagaya and coworkers found reduced infarct size, increased angiogenesis and improved left ventricular function at four weeks in rats given MSC intravenously at 3 hours after coronary artery occlusion, even though only about 3% of the initial dose of 5 million MSC were found in the heart (Nagaya et al. 2004).

Potential importance of timing of cell administration

In the present study cell treatment was given shortly after coronary artery occlusion and just before reperfusion. Recruitment of cells to the site of infarction from the systemic circulation is no doubt affected by factors that change over time. For example, Ma et al. have shown that myocardial stromal cell-derived factor 1, a chemokine suggested to play a role in stem cell homing to damaged tissue, is not maximally expressed until 24 hours after myocardial infarction (Ma et al. 2005). When given immediately after coronary artery occlusion, MSC may locate in the bone marrow and fail to target myocardial damage.

Study Limitations

In the present study, we could not perform histologic analysis to verify the presence of engrafted MSC in the heart and other organs, since the tissue was used for the neutron activation assay. It is therefore possible that the cells died and the europium particles assayed in the tissue represented either free particles in the body or particles phagocytized by macrophages. Terrovitis and coworkers (2008) recently studied stem cells, double labeled with ferumoxide

particles and beta-galactosidase and injected directly into rat hearts. Using magnetic resonance imaging, they found a large ferumoxide signal present in the heart at three weeks, but histologic analysis showed very few beta-galactosidase-labeled cells. The authors concluded that the source of the MRI signal was not from living cells but probably from macrophages, which had engulfed the ferumoxide particles released from dead stem cells. Although we did not perform histologic assessment in the present study, we have published previously (Dai et al. 2005) on histologic data of these same MSC, labeled with DiI, implanted directly into the myocardium. Cells were qualitatively identified by fluorescence microscopy and immunohistochemical staining in myocardial tissue and blood vessels up to 6 months later. We observed DiI positive MSC that co-expressed various muscle markers.

Conclusions

The ability to quantify implanted cells and to determine their distribution in tissues will be important in improving the development of cell delivery and cell retention techniques for use in humans. By using the nanoparticle technique we showed that directly injecting cells into the myocardium improved cell retention to a greater extent than was achieved with intravenous injection followed by cell homing. The nanoparticle technique allowed us to track and quantify the number of retained injected cells. When administered immediately after myocardial infarction in a model of reperfused CAO, direct injection of MSC resulted in 15 % of the labeled cells remaining in the heart one week later. When administered IV, no labeled cells were detected in the heart. Intravenous delivery of cells may not be an effective route of administration as a treatment immediately after myocardial infarction; direct intramyocardial wall injection appears superior. However, it is still possible that stem cells injected intravenously could benefit infarcted myocardium by other so far unknown effects rather than by repopulating the area and creating new myocardium.

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Figure 1.

The relationship between the number of cells and the disintegrations per minute in the standard curve from one expansion. The formula for the relationship is expressed as: (cell number = 1.81 (disintegration per minute) + 3517). The variables were linearly related and showed an excellent correlation ($r = 0.99$)

Figure 2.

Median number of labeled cells detected in liver, lung, spleen and kidney (per gram of tissue) when MSC were administered intravenously or directly into the myocardium. Analyzed by non-parametric statistics (Wilcoxon test) there were no significant differences in the accumulation of cells in these organs based on route of cell delivery.

Table 1

Cells found in the heart

Experiment		Non-infarcted	Infarcted
948	Heart	0	137095
951	Heart	5819	146790
954	Heart	0	151628
957	Heart	0	142669
1033	Heart	115423	294876
1040	Heart	0	308341
1065	Heart	98491	406087
1066	Heart	257429	178550
1071	Heart	0	448503
	MEAN	53018	246023
958	IV	0	0
973	IV	0	0
974	IV	0	0
975	IV	0	0
1039	IV	0	0
1062	IV	0	0
1063	IV	0	0
1038	IV	0	0
	MEAN	0	0

Non-infarcted = non-infarcted portion of the left ventricle (posterior wall + septum), infarcted = infarcted portion of the left ventricle (anterior free wall), heart = cells injected directly into the ischemic risk region of the heart, IV = cells injected via the jugular vein.

Table 2
Techniques to Deliver Stem Cells to Infarcted Myocardium

	Technique	Advantages	Disadvantages
1	Direct intramyocardial injection	Area of interest can be visualized; large number of cells can be implanted directly into the myocardium	Invasive - requires major surgery
2	Transendocardial catheter delivery	Does not require major surgery; can direct cell injection to area of interest using electromechanical guidance; large number of cells can be implanted directly into the myocardium	Requires catheterization laboratory; potential for cardiac perforation or hemorrhage
3	Intracoronary injection	Does not require major surgery; can be performed at the same time as other intracoronary procedures (angioplasty/stenting)	Requires catheterization laboratory; cells may washout of area; potential for coronary embolization
4	Retrograde delivery through catheter in coronary sinus	Does not require major surgery; no concern of embolization	Requires catheterization laboratory; cells may washout area; potential for perforation coronary sinus
5	Intravenous injection	Non-invasive; does not require operating room or catheterization laboratory	Requires that cells "home" to the site of injury; may increase likelihood that cells locate in organs other than the heart
6	Recruitment of endogenous stem cells with humoral factors	Non-invasive; no concern of cell rejection	Mixed results in current preclinical and clinical trials