New Paradigms of Myocardial Regeneration Post-Infarction: Tissue Preservation, Cell Environment, and Pluripotent Cell Sources

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J. Am. Coll. Cardiol. Intv. 2009;2;1-8
doi:10.1016/j.jcin.2008.10.010

JACC: CARDIOVASCULAR INTERVENTIONS VOL. 2, NO. 1, 2009

ISSN 1936-8798/09/$36.00
PUBLISHED BY ELSEVIER INC. DOI: 10.1016/j.jcin.2008.10.010

STATE-OF-THE-ART PAPER

New Paradigms of Myocardial Regeneration Post-Infarction

Tissue Preservation, Cell Environment, and Pluripotent Cell Sources

James S. Forrester, MD, FACC, Anthony J. White, MD, Satoshi Matsushita, MD, Tarun Chakravarty, MD, Raj R. Makkar, MD, FACC

Los Angeles, California

Meta-analyses of intracoronary autologous bone marrow cell infusion in patients with acute myocardial infarction establish the procedure as safe. Nonetheless, the typical small increase in ejection fraction is of uncertain clinical significance, with little if any evidence of myocardial regeneration. In this paper, we describe 3 new paradigms of myocardial preservation and regeneration that provide reasonable hope that the goal of myocardial rejuvenation can be achieved. The first paradigm is that substantial preservation of myocardium is possible even during the period of coronary occlusion and immediate reperfusion, before interventions aimed at myocardial regeneration. The factors that induce myocardial preservation may also create an environment more receptive to subsequent myocardial regeneration. The second paradigm is that the local environment may regulate the behavior of cells in the ischemic/infarct region. For instance, adult cells may be induced to re-enter the cell cycle and proliferate with appropriate environmental modification. The final paradigm is that autologous cardiac stem cells or induced pluripotent stem cells can create new myocytes and myocardium. Taken together, these new ideas, each still to be proven, suggest that the goal of regenerating functioning new myocardium can still be achieved. (J Am Coll Cardiol Intv 2009;2:1–8) © 2009 by the American College of Cardiology Foundation

In 2001, reports that intramyocardial injection of hematopoietic stem cells induced marked histologic and functional improvement in the rodent myocardial infarct model led to worldwide clinical research focused particularly on intracoronary infusion of autologous bone-marrow mononuclear cells (BMCs). The hypothesis that hematopoietic stem cells can give rise to fully differentiated cardiomyocytes, however, was subsequently challenged by several laboratories (1) when labeled hematopoietic stem cells injected directly into ischemic murine myocardium did not express cardiac tissue-specific markers and largely disappeared by 30 days. Later meta-analyses of intracoronary autologous BMC infusion in clinical acute myocardial infarction (MI) suggested that although the procedure induces no increase in late arrhythmias or rehospitalization, the mean absolute increase in ejection fraction is approximately 4% (2). Randomized trials reporting clinical outcomes at 1 year have been similarly mixed. In the REPAIR-AMI (Intracoronary Progenitor Cells in Acute Myocardial Infarction) trial, which randomized 204 patients to intracoronary BMCs or placebo at 3 to 7 days after reperfusion, the composite end point of death, MI, or revascularization was significantly reduced at 12 months (3), but other trials reported an absence of long-term clinical benefit. Most investigators now feel that BMCs may induce paracrine growth factor stimulation or physical scaffolding against cardiac dilation but does not regenerate myocardium (4). Although the mechanisms responsible for failure of myocardial regen-

From Cedars-Sinai Medical Center, Division of Cardiology, Los Angeles, California.

Manuscript received June 13, 2008; revised manuscript received October 6, 2008, accepted October 10, 2008.

eration are many, they may be categorized as related to the cells used, the cells' environment, and barriers created by tissue (Fig. 1). In this paper, we first review the evidence that myocardium can regenerate, and then use 3 paradigms of tissue preservation and regeneration (Fig. 2) to provide a framework for clinicians interested in the research developments that suggest myocardial regeneration in man remains possible.

Evidence That Myocardium Can Regenerate

Myocardial regeneration clearly can occur spontaneously in nonmammalian species. In zebrafish, complete myocardial regeneration occurs within 2 months of amputation of the cardiac apex (5). After fibrin seals the wound, cardiomyocytes infiltrate the clot at its leading epicardial edge and begin proliferating. Bromodeoxyuridine (BrdU) incorporation, a marker of cell proliferation, peaks at about 14 days. The advancing edge of proliferating cells then moves progressively deeper over time, suggesting that regeneration remains localized to the interface with the injured area. Regeneration can be eliminated by a mutation in a mitotic checkpoint kinase (Mps1) that regulates the cell cycle, leading to scar formation typical of infarction in mammals. Collagen deposition, driven by inflammatory cytokines in mammalian species, is not seen in the zebrafish.

Abbreviations and Acronyms

BMC = bone-marrow mononuclear cell
CDC = cardiosphere-derived cell
CSC = cardiac stem cell
MI = myocardial infarction
RISK = reperfusion injury serine kinase
TGF = transforming growth factor

In mammals, however, spontaneous myocardial regeneration has been documented only in the MRL mouse, colloquially called the scarless mouse because of its unique capacity to regenerate tissue after injury. In this mutant animal, initially created for the study of immune deficiency, the histologic response to myocardial injury resembles the regenerative process seen in zebrafish. Leferovich et al. (6) found that cryogen-induced transmural right ventricular infarction in the MRL mouse also heals within 2 months, with restoration of histologically and functionally normal myocardium. The regenerating myocardium exhibits a mitotic index of 10% to 20%, compared with 1% to 3% for control mice. Hydroxyproline levels, a marker of new collagen synthesis, are reduced. Although MRL mouse myocardium clearly has the capacity to regenerate, studies in our laboratory and others showed that after extensive cryoablation and after MI induced by left coronary artery ligation, infarct size is no different from that in nonmutant mice, with a transmural infarction surrounded by collagenous scar. Reviewing these seemingly contradictory results, Vela and Buja (7) suggest that differences in the magnitude of the inflammatory response induced by extensive cryoablation or coronary occlusion compared with limited cryoablation may provide an explanation for these contradictory results. If this postulate is valid, its potential clinical relevance is that the inflammatory environment at 3 to 7 days after infarction may have influenced the effectiveness of prior clinical stem cell trials. If so, then acute preservation of myocardium, an independent goal of ongoing research, may also benefit subsequent therapy directed at myocardial regeneration at 3 to 6 days after the clinical event.

The Paradigm of Myocardial Preservation During Ischemia and Reperfusion

Shortly after occlusion, apoptosis affects more than 80%, and necrosis <20% of the myocytes in the ischemic zone (8). In the first few hours post-occlusion, substantial reduction of apoptotic cell death is possible. The most promising approach to prevention of apoptosis, with concomitant limitation of post-reperfusion inflammation, can be traced back to pre-conditioning studies in which repeated episodes of transient coronary occlusion before permanent coronary occlusion resulted in a marked infarct size reduction (9). For instance, the ratio of infarct size to area at risk was reduced from 48.2% in control animals to 15.1% in rabbits subjected to 2 cycles of 5-min ischemia separated by 10 min reperfusion before 30 min of occlusion and 3 h of reperfusion. Myocardium is also preserved when intermittent occlusion is implemented after reperfusion (termed post-conditioning), with the caveat that the procedure is only effective when implemented immediately post-reperfusion. In the same rabbit model, 6 cycles of 10 s of occlusion separated by 10 s of reperfusion reduced the ratio of infarct size to area at risk to 20.4%. Post-conditioning also has been demonstrated in humans (10). Stented acute MI patients were randomized to either no further intervention or 4 episodes of 1 min balloon inflation followed by 1 min of deflation initiated within 1 min of reperfusion. Creatine kinase release fell by 36%, and angiographic blush grade increased by 25% in the post-conditioning group, with no adverse events. At 6 months the single-photon emission computed tomography rest-redistribution index, a measure of infarct size, was 19.5% in control subjects versus 11.8% in the post-conditioned group, and at 1 year the post-conditioned group exhibited a statistically significant 7% greater left ventricular ejection fraction.

Pre- and post-conditioning can be reproduced by pharmacologic intervention. The conditioning mechanism involves mediators that induce expression and/or activation of serine kinases (nicknamed the reperfusion injury serine kinase [RISK] pathway) that act through closure of mitochondrial transition pores (11) (Fig. 3). When delivered before or soon after coronary occlusion, for instance, adenosine is one such inhibitor of apoptotic cell death. Adeno-

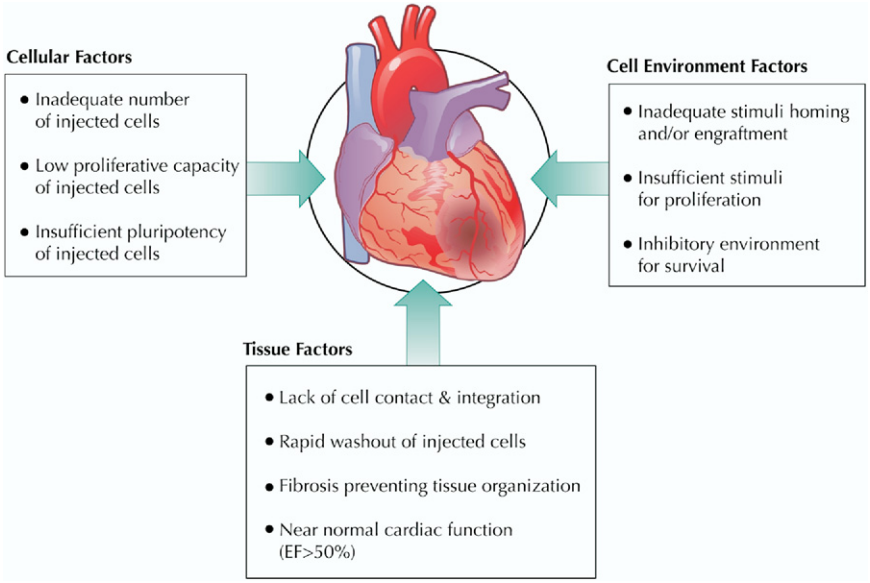


Figure 1. Barriers to Myocardial Regeneration

Barriers to myocardial regeneration exist at the level of the cell, its environment in the adjacent tissue. In addition to these barriers, the optimal timing of stem cell delivery after infarction is, as yet, unknown. Favoring delayed delivery is the natural ebb of acute inflammation over time. This consideration, along with the practical issues of bone marrow cell acquisition and preparation, led to the choice of 6 days as the delay period in clinical trials. Although longer delay could lead to a more stable local environment, increasingly dense collagen deposition might create an insuperable barrier to cell migration and to tissue organization. Figure illustration by Rob Flewell. EF = ejection fraction.

sine also is one of the most potent natural inhibitors of inflammation post-infarct/reperfusion. Recently developed adenosine receptor agonists that are effective in small doses and lack adenosine's undesirable hypotensive or atrioventricular blocking action have this effect. For instance, infusion of an adenosine receptor A_{2A} agonist for 30 min before and after a 90-min occlusion/reperfusion reduced infarct size from 33% to 17% at 48 h in canine MI (12).

A number of clinical trials using pre-conditioning agents that are effective in pre-clinical studies, however, have not resulted in benefit. Most of these trials have 1 of 2 features that may explain the difference from the animal laboratory studies: the agents were infused much later after the onset of coronary occlusion and/or were not given before reperfusion. In this respect, 1 trial of adenosine may provide some insight. Kloner et al. (13) reported the results of anterior ST-segment elevation MI patients randomized to placebo versus adenosine (50 or 70 μg/kg/min for 3 h) starting within 15 min of reperfusion. In patients reperfused <3.2 h after symptom onset, adenosine significantly reduced both 1-month mortality (9.2% vs. 5.2%) and the 6-month composite clinical end point of death, in-hospital congestive heart failure, or rehospitalization for congestive heart failure (17% vs. 12%). In the larger cohort that included patients reperfused beyond 3 h, however, no clinical benefit was seen, although infarct size, as in the animal model, was reduced by about one-half. These laboratory and clinical data suggest that significant preservation of myocardium during coronary occlusion and reperfusion is possible, but that the therapy may have to be given early after coronary occlusion and before or coincident with reperfusion.

A secondary benefit of myocardial preservation is that the intervention may create an environment more receptive to myocardial regeneration therapies. Stem cell engraftment and survival is substantially altered by the local cellular environment. Thus, Laflamme et al. (14) found that whereas 90% of labeled human cardiomyocyte stem cells engrafted in uninjured rat hearts, in only 18% of infarcts were any human cells detected, and the cells that did engraft were found as small clusters (14).

The Paradigm That Local Environment May Regulate Tissue Regeneration

The MRL mouse studies suggest that myocytes may retain the capacity to regenerate depending upon the local environment. The potential importance of a conducive environment is suggested by stem-cell-induced neural regeneration studies in rats with virus-induced hind limb paralysis (15). To overcome myelin-mediated stem cell repulsion, Deshpande et al. (15) used adenosine agonists, then added a neurotrophic factor to attract transplanted embryonic stem cell-derived axons toward skeletal muscle targets. This dual effect of antagonizing

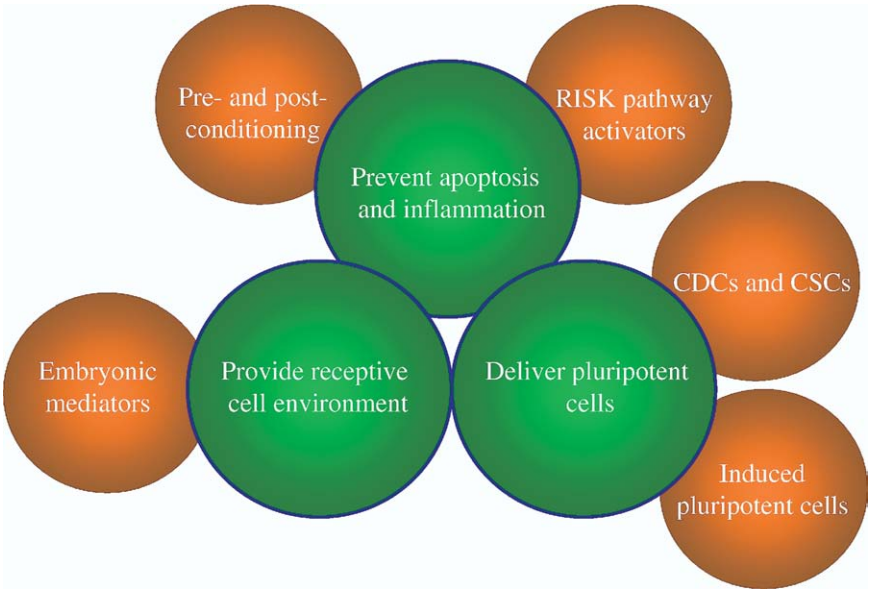


Figure 2. Mechanisms and Means of Preservation and Regeneration

Three mechanisms that may be used to preserve and regenerate myocardium **(green circles)**, and some of the means by which these effects may be achieved **(orange circles)**. Myocardial preservation may be achieved by stimulating the reperfusion injury salvage kinase pathway, using strategies of pre-conditioning and post-conditioning. Myocardial regeneration may be accomplished by stimulating adult cardiomyocytes to re-enter the cardiac cycle, or by delivery of cardiac stem cells (CSCs) or induced pluripotent cells. Each mechanism represents a new paradigm in myocardial preservation and regeneration and, although unproven, is supported by recent animal laboratory research. CDC = cardiosphere-derived cell; RISK = reperfusion injury serine kinase.

the effect of cellular inhibitors combined with stimulation of cell migration and proliferation led to axonal growth, formation of neuromuscular junctions, and restoration of hind limb function at 4 months. No axonal growth occurred with administration of the embryonic stem cells alone. The authors concluded that a dual strategy consisting of suppression of local inhibitors combined with stimulation of cell proliferation was essential for regenerative growth and migration.

Evidence suggesting the existence of 2 potentially competing pathways after injury (i.e., regeneration vs. scarring) also comes from embryologic studies. Mammals early in embryologic development exhibit scarless healing. For instance, epithelial injury in a fetal rat on day 16 exhibits scarless healing whereas the same injury a few days later induces a scar. The transition from scarless healing to scar formation coincides with the appearance of the inflammatory response. Thus, one possible explanation for the transition from scarless embryologic healing seems to be the cell–environment interaction. For instance, scarless embryologic healing is characterized by expression of transforming growth factor (TGF) β3, whereas in scarring TGF β1 and β2 isoforms predominate (16). When TGF β3 is applied to an incisional injury in animals and humans, it markedly reduced and sometimes eliminated incisional scar formation in adult skin and vascular tissue. Taken together, these data suggest that scarless healing may be characterized by reversion of the cell environment to the embryologic state and that this capacity is lost in the adult.

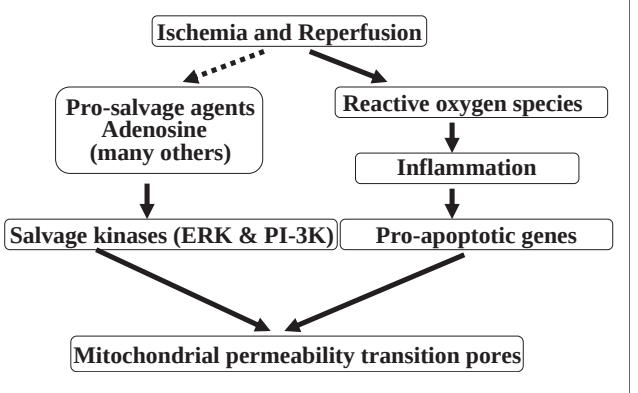


Figure 3. Pathways of Cell Preservation and Cell Death

Pathways of cell preservation and cell death during ischemia and reperfusion. Cell death proceeds through reactive oxygen species, expression of inflammatory mediators, activation of pro-apoptotic genes, and opening of mitochondrial permeability transition pores. Cell survival proceeds through several mediators including adenosine, which activates the expression of pro-salvage serine kinases that oppose the effects of the pro-apoptotic stimuli. The balance between these 2 pathways probably determines cell survival during ischemia. ERK = extracellular signal-regulated kinase; PI-3K = phosphoinositide 3-kinase.

To examine this question, we examined the expression of embryologic transcription factors in peri-infarct tissue of normal mouse hearts. Three factors (Isl1, Mef2c, and HAND1) were up-regulated 2- to 5-fold in infarcted myocardium, accompanied by as much as an 18-fold increase in their respective proteins at 14 days. Periostin expression (vide infra) increased >90-fold. Taken together, these data suggest that a response paralleling cardiogenesis is activated after MI, but that the balance of local factors favors scar formation, and that therapeutic modification of the cell environment might tip the balance to favor regeneration.

Myocardial regeneration by creating an environment that induces adult cells to re-enter the cell cycle. Direct evidence for the influence of the local environment on myocardial regeneration comes from studies of growth mediators known to be important in both embryologic development and wound healing. For instance, p38 mitogen-activated protein (MAP) kinase plays a central role in the transition from embryologic to adult cell behavior by activating genes that inhibit cell division. In cell cultures, Engel et al. (17) found that inhibition of p38 MAP kinase induces in a marked increase in cell dedifferentiation and a 92% increase in myocyte proliferation. Extending these studies to the animal MI model, they found that when a p38 MAP kinase inhibitor was combined with fibroblast growth factor to stimulate myocyte and endothelial cell proliferation, scar volume significantly fell (from 42% to 24%) and fractional shortening significantly improved (from 34% to 53%) at 3 months post-infarction. Interestingly, p38 MAP kinase inhibitors and other mediators with similar effects are antiapoptotic and anti-inflammatory.

Periostin is an embryonic factor that is reactivated in the adult after tissue injury. In myocyte cell culture, extracellular periostin induced differentiated mononucleated mammalian cardiomyocytes to re-enter and complete the full mitotic cell cycle. Interestingly, the action of periostin required the presence of one of the members of the RISK pathway (phosphoinositol 3-OH kinase) previously identified as central to pre- and post-conditioning, suggesting the possibility that activation of the RISK pathway during preservation might also contribute to subsequent therapy aimed at regeneration. Extended to the rat infarct model, periostin resulted in improved ventricular remodeling and myocardial function, reduced fibrosis and infarct size, and increased angiogenesis, suggesting that it may be possible to induce existing adult cardiomyocytes to proliferate, given the appropriate cellular environment. In summary, the data suggest a new concept for myocardial regeneration. Whereas prior dogma has been that adult cardiac cells are postmitotic and incapable of regenerating myocardium, these data suggest that at the time of infarction, the cells may induce either scar or regeneration depending, in part, upon the cellular environment. The factors thus far identified to have the capacity to induce cell dedifferentiation are also antiapoptotic and some are known mediators of preconditioning, providing a potential link between the strategies of prevention and regeneration.

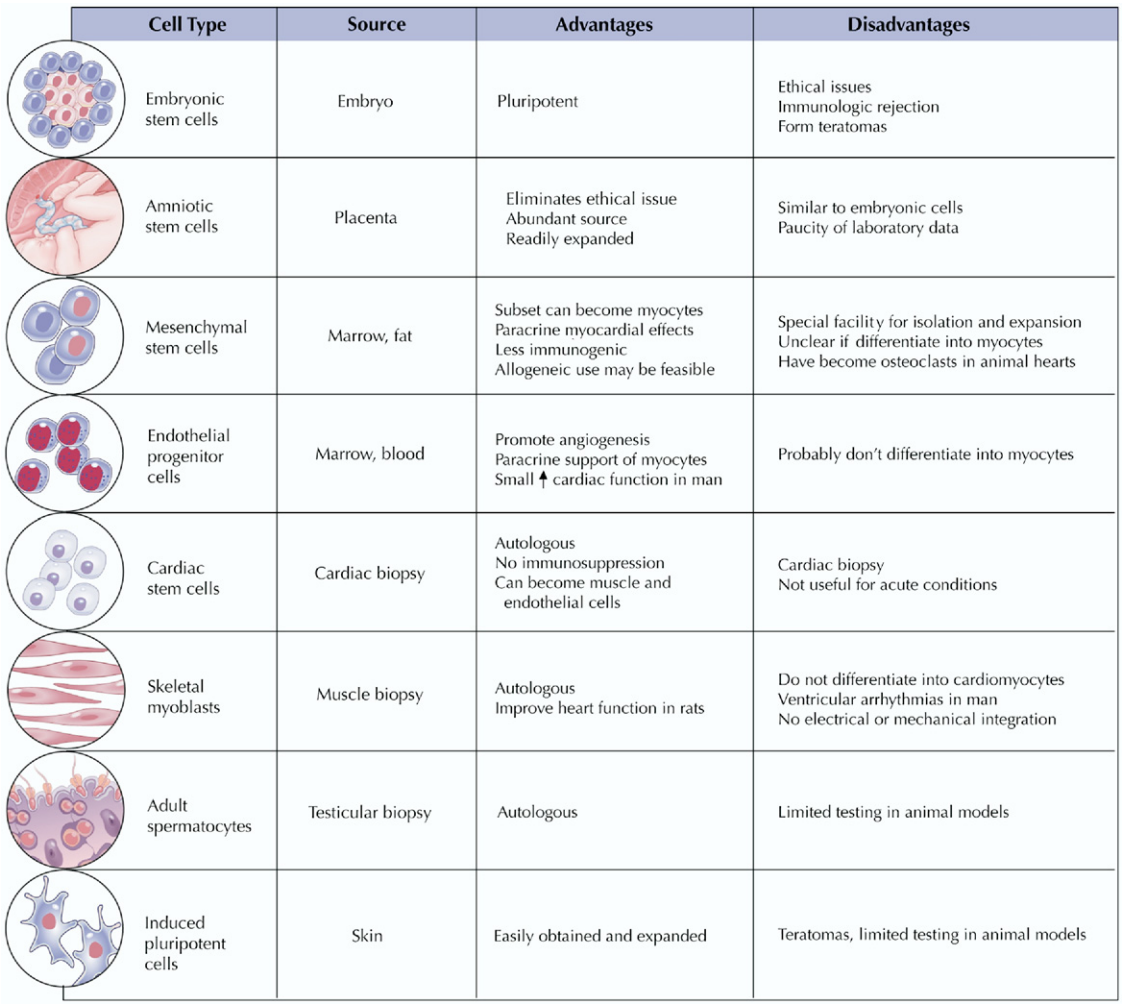
The Paradigm That Regeneration May Be Induced by Cardiac Stem Cells (CSCs) or Induced Pluripotential Cells

An alternative paradigm is that exogenous primitive cells can be induced to become functioning adult cardiac cells. A variety of primitive noncardiac cells and methods of cell delivery (19–22) reduce apoptosis, increased capillary density, and improve ejection fraction in infarct models. Since BMCs have produced weak clinical results, alternative cell types are now under investigation. The advantages of cell types other than BMCs are shown in Figure 4.

Myocardial preservation using autologous CSCs. In 2002 autopsied female donor hearts transplanted into male recipients were found to have myocytes, coronary arterioles, and capillaries with a Y chromosome making up 7% to 10% of the cells. Subsequently, these highly proliferative CSCs were identified in normal hearts, typically located in interstitial niches of the atria and ventricles (23). Although the origin of these cells remains unclear, the niche serves an anatomic compartment in which stem cell self-renewal, protection from cell death, and the fate of stem cell daughters is regulated by integration of intrinsic cell factors with the niche microenvironment. This discovery represents a major paradigm shift since the heart was thought to be a post-mitotic organ without the capacity to replace itself. The new paradigm suggests that apoptotic cardiac cells are slowly replaced by new cells derived from CSC.

Several laboratories have now shown that injection of previously isolated and expanded CSCs can partially restore regional myocardial structure and function in the animal MI model. For instance, Dawn et al. (24) delivered CSCs by aortic root injection in a 90-min occlusion/4-h reperfusion rat MI model. Treated hearts had a smaller left ventricular chamber volume and a greater wall thickness in the infarct area compared with control animals at 5 weeks. The injected CSCs induced myocardial regeneration and decreased infarct size by 29%.

Creation of sufficient numbers of CSCs to support regeneration. A potential issue for use of autologous CSCs in man, however, is the number of cells to be delivered. At least 2 successful approaches to generation of high-volume autologous CSCs are now being pursued, with the goal of clinical testing. Bearzi et al. (25) identified, isolated, and expanded human CSCs from surgical myocardial biopsies of 8 patients, then injected the cells into the infarcted myocardia of immunodeficient rodents. The injected cells generated a chimera with human myocytes, coronary resistance arterioles, and capillaries. Areas of myocardial regeneration within the infarct were found in 17 of 25 treated mice



Cell Type	Source	Advantages	Disadvantages
Embryonic stem cells	Embryo	Pluripotent	Ethical issues Immunologic rejection Form teratomas
Amniotic stem cells	Placenta	Eliminates ethical issue Abundant source Readily expanded	Similar to embryonic cells Paucity of laboratory data
Mesenchymal stem cells	Marrow, fat	Subset can become myocytes Paracrine myocardial effects Less immunogenic Allogeneic use may be feasible	Special facility for isolation and expansion Unclear if differentiate into myocytes Have become osteoclasts in animal hearts
Endothelial progenitor cells	Marrow, blood	Promote angiogenesis Paracrine support of myocytes Small ↑ cardiac function in man	Probably don't differentiate into myocytes
Cardiac stem cells	Cardiac biopsy	Autologous No immunosuppression Can become muscle and endothelial cells	Cardiac biopsy Not useful for acute conditions
Skeletal myoblasts	Muscle biopsy	Autologous Improve heart function in rats	Do not differentiate into cardiomyocytes Ventricular arrhythmias in man No electrical or mechanical integration
Adult spermatocytes	Testicular biopsy	Autologous	Limited testing in animal models
Induced pluripotent cells	Skin	Easily obtained and expanded	Teratomas, limited testing in animal models

Figure 4. Potential Cell Sources for Myocardial Regeneration: Advantages and Disadvantages

Among the most important advantages for individual cell types are the capacity to differentiate into all cardiac cell types and an autologous origin. Among the most important potential disadvantages are the need for immunosupression, potential for tumor formation, and difficulty of cell acquisition. Figure illustration by Rob Flewell.

(68%), and 14 of 19 treated rats (74%). Human myocardium comprised 1.3 mm^3 in mice and 3.7 mm^3 in rats, of which approximately 84% were myocytes, and 8% were vascular structures. Remarkably, the human tissue was sufficiently integrated that it contributed to the regional and global function of the recipient heart. The CSCs were isolated by antigenic selection using antibodies against c-kit, the receptor for stem cell factor. This requirement for exposure to xenogenic antibodies could pose a hurdle to regulatory approval for clinical trials involving c-kit+ CSCs.

An alternative approach is the use of cardiosphere-derived cells (CDCs), which do not require use of antibodies for selection. Smith et al. (26) cultured cardiac muscle samples from endomyocardial biopsy specimens. The primary culture developed multiple spherical clusters termed cardiospheres, which were then plated to yield CDCs. Human CDCs were cultured from endomyocardial biopsies in 69 of 70 patients. In vitro studies demonstrated that the CDCs are cardiogenic. Injected into the border zone of acute MIs in immunodeficient mice, both the percentage of viable myocardium within the infarct zone and the ejection fraction were greater in the CDC-treated group than in the fibroblast-treated control group at 20 days. Myocardial regeneration was documented histologically as an increase in the amount of viable tissue within the infarct zone. It is possible that this approach to cardiac cell therapy may be approved for initial clinical testing within the coming year.

Modification of the local cell environment is likely to be a critical adjunct to stem cell therapy. To test this hypothesis, Laflamme et al. (14) created a cocktail of 6 pro-survival

factors that were delivered with human embryonic stem-cell-derived cardiomyoctes in the rat infarct model. Compared with stem cells alone, the cells of the treated animals developed much larger segments of human myocardium within the infarcted area, and exhibited a 2.5-fold increase in regional wall thickening (14).

The potential for cardiac regeneration using autologous-induced pluripotent cells. In the less immediate future is the possibility that autologous pluripotent cells can be derived from adult noncardiac tissue. Guan et al. (27) recently reported the creation of pluripotent stem cells with phenotypic characteristics of embryonic stem cells from adult mouse testes. These cells are capable of spontaneous differentiation into cells of all 3 germ layers. With manipulation of the cell culture environment, the cells were induced to differentiate into functional cardiomyocytes that expressed cardiac-specific L-type calcium (Ca^{2+}) channels and responded to Ca^{2+} channel-modulating drugs. The cells also formed functional gap junctions, and when beating they exhibited rhythmic Ca^{2+} transients. The beating frequency was responsive to stimulation with isoproterenol, and suppression by cadmium chloride abolished spontaneous electrical activity. When transplanted into a normal mouse hearts, the cells were able to proliferate and differentiate. Patient-specific autologous cardiomyocytes derived from testicular biopsy could circumvent the ethical and immunological problems associated with human embryonic stem cells.

Two laboratories have shown that pluripotent human cells similar to embryonic stem cells also can be created from dermal fibroblasts by retroviral introduction of 4 transgenes. Two of the transgenes are the same in both laboratories (Oct3/4, Sox2) and 2 are different (28,29). These studies suggest that transcription factor-induced nuclear reprogramming can lead to the reversion of somatic cells to an embryonic cell-like state. Placed in an appropriate environment (activin A and bone morphogenic protein), these cells can develop into beating cardiomyocytes. The requirement for genetic modification may dampen the prospect for rapid regulatory approval of ethical trials since at least some of these cell lines have the capacity to induce teratoma formation. As a consequence, other lines of research are focusing on methods of cell selection that allow creation of pluripotent cells that lack this capacity.

Approaches to improving stem cell engraftment and persistence. Independent of the cell source is an additional problem of inadequate cell engraftment, persistence, and potentially inhomogeneous cell distribution. One potential solution, at least during surgery, is the use of a synthetic matrix (30). Both gelfoam and biologic matrices are being tested. For instance, in one such application, human mesenchymal stem cells were embedded into a tissue-engineered cardiac patch containing a rat tail type I collagen matrix, and attached with fibrin sealant to the epicardial surface of immunocompromised rat anterior MI. When the patches were applied after 4 days of culture, >90% of cells were viable. At 1 week, 23% of mesenchymal cells had engrafted. At 4 weeks, anterior wall thickness increased and fractional shortening increased by 30%. Patches alone induced no improvement. Nonetheless, in the treated animals, mesenchymal cells were not detectable at 4 weeks, suggesting that although improvement did not require long-term cell engraftment, persistence of cells in host tissue may require more than purely mechanical solutions.

Conclusions

Clinical trials of intracoronary delivery of autologous BMC infusion in acute MI have established safety and feasibility with only a modest effect on cardiac perfusion and function, and with no evidence for creation of new myocardium. These equivocal results have been paralleled by 3 new still-to-be-proven paradigms that suggest that with perseverance the goal of preserving and then regenerating functioning new myocardium can still be achieved in man.

Reprint requests and correspondence: Dr. James S. Forrester, Burns and Allen Professor, Division of Cardiology, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Los Angeles, California 90048. E-mail: forrester@cshs.org.

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Key Words: stem cell ■ cardiosphere-derived cell ■ bone marrow cell ■ myocardial regeneration ■ myocardial preservation.

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J. Am. Coll. Cardiol. Intv. 2009;2;1-8
doi:10.1016/j.jcin.2008.10.010