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Cardiac Regeneration

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Introduction

Cardiovascular disease is a generic term that refers to any illness or disorder that involves the heart and its vessels or the blood vessels of the body. Cardiovascular disease has been accepted as the leading cause of death worldwide. According to the Centers for Disease Control's National Vital Statistics Reports, twenty six percent of deaths in 2006, 631,636 in total, were caused by diseases of the heart (Heron et al. 2009).

One of the most common of all cardiovascular diseases is Ischaemic Heart Disease. This coronary artery disease often leads to Acute Myocardial Infarction, more commonly known as a heart attack. An ischemia occurs when an organ is receiving an insufficient supply of blood, often caused by a clogged artery (De Milto 2006). Atherosclerosis and blood clots in some of the larger coronary arteries are the most common condition to block coronary circulation. Coronary arteries take rise from the root of the aorta and spread out over the epicardium. These arteries branch out into energy hungry cardiac muscle, the myocardium, to supply it with oxygen and nutrients. The blockage of these deep arteries is known as myocardial ischemia. When ischemia to a specific region is severe, injury occurs (American Heart Association 2008). When blockage results in cell death, the condition is called an infarction.

Myocardial infarction is characterized by necrosis, cellular death in an isolated region of the heart. Cellular death stimulates the migration of macrophages to the infarct zone. They remove the necrotic tissue, and the area is refilled with a dense collagen scar, leading to a decrease in muscle thickness. The scar tissue, by and large, is acellular, and does not have the biochemical properties of myocardial cells. This can lead to electrical inconsistencies, loss of structural integrity, and mechanical dysfunction, such as arrhythmias. Together, these abnormalities can lead to complete heart failure (Joggerst and Hatzopoulos 2009).

Current treatments for post myocardial infarction patients aim to assist in the healing process and prevent further heart attacks, such as cholesterol-lowering medications and angiotensin inhibitors. A rehabilitation plan that includes blood pressure and cholesterol management may be set up. Diet and exercise regimes may change (Mayo Clinic 2010). Ultimately, though, once an area of the myocardium is dead, it's irreversible. It is because of this that scientists are looking for ways to alleviate the effects of myocardial ischemia and infarction by means of cardiac regeneration.

Cell Therapy

The basic concept of cell therapy is that exogenous, or possibly even autologous cells implanted into diseased tissue can recover and improve its function by substituting for the resident cells that have died. The improved neurologic function of Parkinson's patients that underwent the grafting of intracerebral dopaminergic cells and the prospect of weaning a number of diabetics off insulin after Langerhan islet transplants provide a good indication that cell therapy can be clinically effective. Transplanted cells must be engrafted in very specific quantities, and they must be able to integrate into normal host tissue without excess reproduction

and other adverse effects. When attempts to ameliorate the effects of myocardial infarction by converting the infarct zone into contractile tissue with the use of viral vectors encoded with muscle specific master genes failed, cell therapy became an increasingly attractive method of attempted cardiac regeneration (Menasche 2004).

Stem Cells:

Stem cells can be defined by two significant characteristics. First and foremost, they must be able to self-renew. They must be able to go through many cycles of mitosis and still remain in their undifferentiated state. Secondly, a stem cell must have the capability to differentiate into various specialized cell types. The effectiveness and value of stem cells are often based on their potential to do this. Pluripotent stem cells are able to differentiate into specialized mature cells from the three germ layers. The embryonic stem cell is the classic example of a pluripotent stem cell. Multipotent stem cells, alternatively, are limited to differentiating into mature cells of directly related lineages, generally from the same germ layer. Adult stem cells, sometimes called somatic stem cells, that are derived from postnatal, or even fully mature tissue are generally multipotent. Some adult stem cells sometimes have a more limited capacity for self-renewal, and are committed to differentiation into specific lineages. These cells are called adult progenitor cells (Cook et al. 2009). Until very recently, the myocardium has been labeled and viewed as an end-differentiated organ – one without any potential for regeneration. Certain types of stem cells are currently being tested as sources for regenerative potential for the heart. There is a new availability for hope in the possibility of reconstructing the infracted, failing ventricle (Loscalzo et al. 2008).

Mesenchymal Stem Cells:

Mesenchymal stem cells (MSCs) were first identified by their ability to form fibroblast like populations, and have been recognized for their plasticity. In the past, they have been known to help normal bone regenerate in vivo. They were initially discovered in the stroma of bone marrow. It has been established that by secreting growth factors important for proliferation and colony stimulating factors, MSCs provide support for hematopoiesis. While generally harvested from bone marrow, MSCs have recently been found in and isolated from the placenta, adipose tissue, the liver, and umbilical cord blood. MSCs, an integral part of the composition of endothelium (perivascular cells), are now seen as ubiquitous. No single, specific marker for MSCs has been discovered yet, though they are characteristically lacking typical hematopoietic antigens, such as CD 34, CD 44 an45, and more. MSCs produce a large number of cytokines and growth factors, including the important vascular endothelial growth factor . The hallmark and most distinctive feature of the mesenchymal stem cell is its inherent ability to differentiate into anything of mesodermal lineage, such as bone, cartilage, tendon, muscle, and adipose tissue. The main advantage of MSCs in cell therapy is their unique immunological properties. They can be transplanted across major histocompatibility barriers; recipients will not need to take immunosuppressants. They can be taken from any healthy donor, even an unrelated one, and cryopreserved until needed. With their ability to self-renew and differentiate, MSCs can be induced to differentiate into cardiomyocytes, and there is evidence that MSCs migrate primarily to sites of inflammation. These factors together suggest their potential for treatment for ischemic myocardial infarction. A disadvantage of MSCs is that they are perhaps too heterogenic, and

there are concerns that they may provide unexpected results, such as ossification (Cook et al. 2009).

Intracoronary injections of autologous bone marrow have led to moderate recovery of cardiac function, but clinical findings show that bone marrow cells don't actually engraft in the infarct zone, nor do they reduce infarct size. A placebo controlled study was therefore done to increase the evidence that MSCs have reparative properties. To ensure that mesenchymal stem cell grafts were allogeneic, donor pigs were of a different strain than recipient pigs. The bone marrow was obtained from the iliac crests of male swine. MSCs were identified and isolated by density, and were plated to expand in culture, and were then cryopreserved. They were magnetically stained in order to be later identified under MRI. Recipient pigs underwent a surgically induced myocardial infarction via balloon occlusion of the left anterior descending coronary artery for sixty minutes, followed by reperfusion; left ventricular performance and cardiac oxygen consumption was measured. After three days, animals were randomly chosen to receive intramyocardial infusions of allogeneic porcine MSCs or a placebo through a needle tipped injection catheter. Contractility of the myocardium was measured by the maximal rate of isovolumetric contraction and ventricular elastance, and the ratio of left ventricular systolic pressure to stroke dimension. At day three post injection, no animal had died or shown signs of arrhythmias, or shown signs of cardiac perforation, thus indicating that the method of delivery was safe. The animals were euthanized after eight weeks. Their hearts were analyzed in gross and microscopic levels. Tissue samples were taken from the infarct zone, the infarct border, and remote tissue. Immunostaining revealed the presence of α-actinin, troponin-T, and myosin heavy chains. Histologic evaluation demonstrated that MSCs were found present throughout the infarct and border regions, and they expressed muscle specific proteins that weren't found while they were still in culture plates. They were also found in vascular structures, where they had incorporated into vascular smooth muscle and endothelium. Myocardial infarction normally begins at the subendocardial area of the myocardium and moves out through the midmyocardium out towards the subepicardia

Table 1. Hemodynamic measurements

		8 weeks post-MI	
	Normal	Placebo	MSC
LV end-diastolic pressure, mm Hg	8.4 ± 2.3	29.8 ± 7.6	20 ± 6.4†
LV end-systolic pressure, mm Hg	107.1 ± 4.2	117.7 ± 22.2	113.8 ± 5.8
Arterial elastance, mm Hg/mm	14 ± 2	26.1 ± 8.7	17.1 ± 3
dP/dt_{max}, mm Hg/s	2,560 ± 266	1,720 ± 351	2,465 ± 574†
Ees, mm Hg/mm	16.3 ± 2.4	7.9 ± 1.2	17.1 ± 2†
τ, ms	36.2 ± 1.8	52.6 ± 11.6	34.2 ± 1.2†
SW, mm Hg/mm	771 ± 116.5	470.3 ± 86.9	654.4 ± 129.3†
MVO_2, J per beat	3.2 ± 0.9	12.9 ± 1.4‡	3.7 ± 1.8‡
SW/MVO_2	9.1 ± 1.6	2.9 ± 0.1‡	10 ± 5.6‡

MVO_2 indicates myocardial oxygen consumption and SW/MVO_2 myocardial efficiency.
*$P < 0.05$ vs. normal (pre-MI).
†$P < 0.05$ vs. placebo (2-way ANOVA).
‡Listed values were measured at 4 weeks.

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region. In animals that received a mesenchymal stem cell transplant, the infarct region was confined to the midmyocardium. When measured, the subendocardial rim was thicker in the mesenchymal stem cell-treated group than in control groups. MSCs probably caused this cardiac regeneration of the subendocardium. The effects of the transplant on cardiac function are as follows (see table 1).

The animals exhibited recovery to almost normal levels of both systolic and diastolic function. Myocardial efficiency increased, almost bringing stroke work back to a normal level. Cardiac energy metabolism was nearly recovered (Amado et al 2005).

Hematopoietic Progenitor Cells /Bone Marrow Mononuclear Cells:

Hematopoietic progenitor cells are the only stem or progenitor cell that are routine in clinical use today, due in part to the fact that they are easy to isolate and feasible to implant (Joggerst and Hatzopoulos 2009). They reside in bone marrow, and are responsible for making all blood cells, constantly repopulating the hematopoietic and immune systems. They are used in bone marrow transplants to treat many disorders, including leukemia and aplastic anemia. In humans, hematopoietic progenitor cells are identified by the CD 34 marker (a surface gylocoprotein). Hematopoietic progenitor cells home to bone marrow in healthy mammals, where they adhere firmly to the endothelium. Whether or not hematopoietic progenitor cells actively participate in the repair of cardiomyocytes after an infarction is a matter of debate, though they have been known to differentiate into skeletal muscle fibers. It is generally accepted, though, that hematopoietic progenitor cells are responsible for the inflammatory wound healing process (Cook et al. 2009). Experimental studies have suggested that intramyocardial or intravascular administration of bone marrow derived he (Amado et al. 2005) progenitor cells may play a part in the functional regeneration of infarcted c enhancing neovasculogenesis of an ischemic myocardium (Schachinger et al. 2006).

Patients, aged eighteen to eighty, were eligible for the REPAIR-AM Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction) trial if they h had an ST-elevation myocardial infarction with residual left ventricular wall motion abnormality and a significantly decreased left ventricular ejection fraction (Schachinger et al. 2006). (A normal resting ejection fraction, the fraction of blood pushed out of the heart by the ventricles per beat, is 62.3±6.1% [Pfisterer and Battler 1985].) This placebo controlled, double blind randomized trial was performed in seventeen different centers, at a median of 4 days after AMI reperfusion therapy. Bone marrow biopsies were done on 204 patients and the aspirate was sent to a single cell-processing laboratory, where patients were randomized to receive either an intravascular infusion of bone marrow derived hematopoietic progenitor cells (101 patients) or a placebo medium (103 patients). This infusion was done using a stop-flow technique via balloon positioned in the infarct-related coronary artery. After four months, the BMC (bone marrow cells, and alternate name for hematopoietic progenitor cells) group showed enhanced contractile recovery of left ventricular function. One year later, the results of the experiment were measured. A total of eight deaths occurred in the year, six in the placebo group and 2 in the BMC group. None of the patients in the BMC group experienced a second myocardial infarction, while six patients in the placebo group suffered a total of eight myocardial infarctions, six of which were located to the target blood vessel. While on paper these numbers may seem small, statistically, the difference is significant. Similarly, additional revascularization procedures were needed less frequently in the BMC group than in the placebo group. Thirty-

eight revascularizations were needed in thirty seven patients of the placebo group, while only twenty five were needed in twenty two patients of the BMC group (Schachinger et al. 2006).

The BMC group can be seen as a predictor of a reduced cardiovascular event rate. It is reassuring that in every endpoint, be it death, another myocardial infarction, or rehospitalization due to heart failure, there was a trend, albeit a statistically insignificant one, in favor of the BMC group. This data suggests that the contractile recovery seen at four months may possibly translate into a better clinical outcome one year post BMC infusion (Schachinger et al. 2006).

Cardiac Stem Cells:

The steps that have been made towards cardiac regeneration through the various stem cell types prompted further research into any natural regenerative properties and mechanisms that cardiac tissue might have. The heart has always been viewed as a postmitotic organ because mature cardiomyocytes don't reproduce or propagate. Contradictory facts seemed to accumulate as cardiomyocytes were discovered proliferating and reentering the cell cycle in certain pathological conditions such as ischemia and hypertension (Joggerst and Hatzopoulos 2009). After a female heart was transplanted into a man, male cardiomyocytes and endothelial cells were found growing within the heart. Y-chromosome positive cells must have migrated from the recipient's atrial stump or bone marrow into the donated heart, and differentiated into

Table 2
Clinical events during 1-year follow-up

Number of patients with events	Placebo[a] (*n*=103)		BMC (*n*=101)		*P*-value
	n	%	*n*	%	
Death	6	5.8	2	2.0	0.28[b]
Cardiac death	4	3.9	2	2.0	
Myocardial rupture	1	1.0	1	1.0	
Myocardial infarction	1	1.0	0		
Sudden death	1	1.0	1	1.0	
Heart failure	1	1.0	0		
Cardiovascular death (stroke)	1	1.0	0		
Non-cardiovascular death (cancer)	1	1.0	0		
Myocardial infarction	6	5.8	0		0.029[b]
Rehospitalization for heart failure	3	2.9	0		0.25[b]
Revascularization	37	36	22	22	0.026[c]
Target vessel revascularization	26	25	16	16	0.097[c]
Stent thrombosis	3	2.9	1	1.0	0.62[b]
Non-target vessel revascularization	16	16	7	6.9	0.052[c]
Documented ventricular arrhythmia or syncope	5	4.9	5	5.0	1.0[b]
Ventricular arrhythmia	4	3.9	5	5.0	0.75[b]
Syncope	1	1.0	0		1.0[b]
Stroke	1	1.0	1	1.0	1.0[b]
Cancer	2	1.9	0		0.50[b]
Combined events					
Combined death or myocardial infarction	10	9.7	2	2.0	0.019[c]
Combined death, infarction, or any revascularization	42	41	24	24	0.009[c]
Combined death, infarction, or infarct vessel revascularization	31	30	18	18	0.040[c]
Combined death, infarction, or rehospitalization for heart failure	12	12	2	2.0	0.006[c]

[a]In 3 patients, only the 4 months follow-up was available.

[b]Fishers exact test.

[c]Chi-square test.

(Schachinger et al. 2006)

functional cardiomyocytes (Quaini et al. 2002). Since then, several types of cells have been discovered in the adult heart that have stem cell characteristics. One stem cell characteristic is a cytoplasmic exclusion of certain vital dyes, like Hoechst 33342 and Rhodamine 123. Populations of these cells, sometimes called side population cells, have been found in various organs, such as skeletal muscle, bone marrow, and adipose tissue. These cells have recently been found in cardiac tissue as well, concentrated in the deep tissue of atria and apex. Cardiac side population cells have been seen to differentiate into cardiomyocytes, suggesting that they may in fact be cardiac progenitor cells. After cardiac injury, such as ischemia, these cardiac side population cells are mobilized and move to the injury site. The apparent conflict between the existence of cardiac progenitor cells and the heart's lack of regenerative ability is still puzzling to researchers, though two theories have been developed. The first is that these cells, along with mature cardiomyocytes, cannot survive the hypoxic conditions of ischemia. The second is that the pool of cardiac stem cells diminishes with age, which can possibly contribute to lack of regeneration in the elderly (Joggerst and Hatzopoulos 2009).

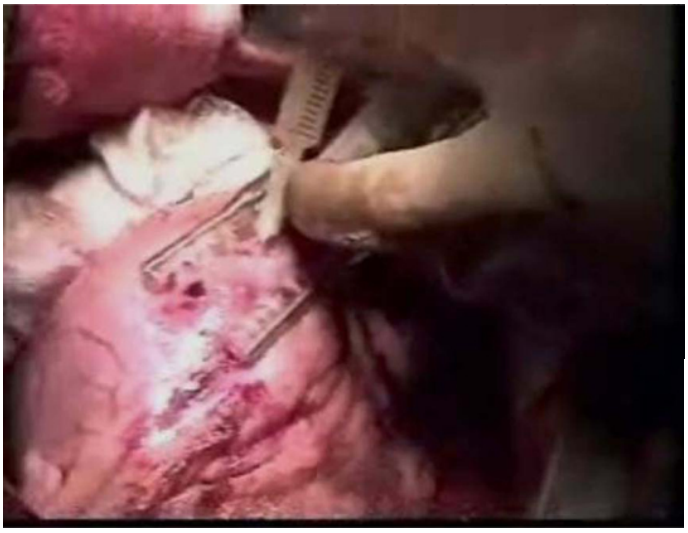
Studies have found what have been loosely termed human cardiac stem cells in small niches in the heart. These small clusters of human cardiac stem cells are closely connected to myocytes and fibroblasts by gap junctions and adherans junctions. The myocytes and fibroblasts form supporting walls that contain the cardiac stem cells. These cells are very obviously committed to myocyte lineage. Stem cell antigen C-kit^{POS}, sarcomeric proteins, and myocyte transcription factors are co-expressed consistently, though they sometimes also expressed transcription factors for endothelial cells and smooth muscle cells. Myocardial samples were enzymatically dissociated, and C-kit^{POS} cells sorted out and plated; multicellular clones were formed successfully in eight out of twelve cases. These clones were, in turn, plated again, or placed in individual wells. Doubling time was approximated at 29 hours. These cells differentiated into cardiomyocytes, smooth muscle cells, and endothelial cells. Developing myocytes had sarcomeric units, were striated, and showed contractile activity after electrical stimulation (Bearzi and Rota 2007).

Human cardiac stem cells were collected from eight patients and injected into infarcted mouse or rat heart, forming chimeric organs that contained human myocytes and coronary vessels. Cell therapy led to regeneration in the infarct zone. The new cells tested positive for α-sarcomeric actin and human DNA sequences were found in seventeen out of twenty five treated mice and fourteen out of nineteen rats. Of the new tissue, ≈84% was myocardial and ≈8% consisted of new microvasculature, making an approximate ration of one capillary to eight cardiomyocytes. Two different lentiviruses were injected into the cardiac stem cells and the infarcted rat and mice hearts to promote different dye production, and the results showed that there had been no cell fusion. The new cardiomyocytes were all human, and that animal cardiomyocytes did not contribute to the regeneration or repair. An examination of ECG done on injected hearts showed that tissue regeneration had partially restored contractile function in the infarct, resulting in an increased ejection fraction and a general improvement of ventricular function. When examined, the synchronicity of calcium tracings in both human and rat cardiomyocytes proved their functional integration. An occasional protein was even found linking human and rodent cardiomyocytes. Together, these observations all prove the role of transplanted human cardiac stem cells in cardiac homeostasis and myocardial regeneration (Bearzi and Rota 2007).

Skeletal Myoblasts:

While the regenerative ability of cardiac muscle cells is a relatively new development, it has been known for a while that skeletal myoblasts retain the potential to regenerate. This is due in large part to the presence of stem cells, sometimes called satellite cells, in this case, because of their location in the periphery of mature, multinucleated muscle tubules. A billion or more myoblasts can be easily cultured in the laboratory from a single small piece of muscle tissue. Myoblast therapy was developed originally as a possible treatment for muscular dystrophy, because myoblasts from a normal donor would fuse with the muscle of the recipient to provide normal proteins absent from dystrophic patient. In 1992, scientists realized the potential use of skeletal myoblasts in cardiac regeneration. Over time, experiments proved that myoblast transplantation could repair a damaged myocardium when cells engraft and integrate, adding new contractile muscle to the heart, and by promoting cardiac repair, including rejuvenation of blood supply, and therefore oxygen and nutrients, to previously damaged areas (Dinsmore and Nabil 2006).

Of one the wonderful things about myoblast therapy is that it can be autologus. In preparation for transplant, a skeletal muscle biopsy about five grams is done. The muscle is trimmed of all connective tissue and minced. It then goes through several cycles of digestion with trypsin and collagenase. The resulting cells are plated and grown in a medium developed primarily for supporting myoblast growth. The actual number of cells used for transplant can be anywhere from three hundred million to eight hundred million. Animal studies done with transplantation of skeletal myoblasts into ischemically damaged myocardium have demonstrated good results. They show that skeletal myoblasts engraft and contribute to improved heart function; they have survived long term without manifesting adverse affects, such as arrhythmias. Autologus rat myoblasts grafted into ischemic myocardium survived both inside and out of the infarct zone, and fused, forming myotubes in close contact with myocytes at the border of the infarct zone. Myocardial contractility and cardiac output increased in comparison to the control group. Ventricular volume and remodeling were minimal. In sheep, progressive heart failure halted short term , and was functionally reversed in the long term. Skeletal muscle fibers formed organized bundles that co-aligned with adjoining cardiac muscle. Muscle density in areas of ischemic collagen scars was almost restored to normal. Clinical studies have been done in both the USA and Europe, and direct evidence for skeletal myoblast survival had been provided. Autologus myoblasts formed myofibers that survived in the human myocardium and aligned



Autologous human myoblast transplantation in a human patient as an adjunct to coronary artery bypass surgery on a beating heart (Haider et al. 2004).

parallel to host cardiac muscle fibers, showing potential for synchronized contraction. This in turn, can contribute to improvements in systolic and diastolic functioning. Left ventricular ejection fraction increased. Tissue viability scanning of the scarred region of the myocardium six months post transplant showed improved metabolic activity (Dinsmore and Nabil 2006). While the theory shows great promise, a few problems with myoblast transplantation were exhibited in some trials. Ten patients that received myoblast transplants reported severe left ventricular dysfunction. Their ejection fraction dropped significantly to below 35%. In the MAGIC (Myoblast Autologuous Grafting in Ischemic Cardiomyopathy) trial, four out of sixty four patients developed ventricular tachycardia after receiving skeletal myoblast injections in coronary artery bypass grafting, and twenty four more were expected to. Therefore, clinicians

must proceed with caution in regard to arrhythmias in pursuing myoblast therapy. Furthermore, many myocardial infarction patients are not surgical candidates, and a less invasive method of delivery must be devised (Dinsmore and Nabil 2006).

Of all the various studies done to investigate the effects of cell therapy on myocardial infarction, few have taken timing into account, and as of yet, the optimal time for cell transplantation in heart attack patients remains unclear. One such study was done in 2009 using human umbilical cord blood cells (HUCBC), which are rich in mesenchymal progenitor cells and contain endothelial cell precursors with huge in vitro proliferation capabilities. Myocardial infarction was identified in eighty male Wistar rats by elevated ST segments in a transthoracic ECG. Equal volumes of either HUCBCs or a phosphate buffered saline placebo were injected into the rats through a caudal vein at days one, five, ten, and thirty after myocardial infarction. The rats were all sacrificed four weeks post infusion, after a hemodynamic assessment and an ECG. Mortality in rats transfused on day one post infarction was 40%, most deaths occurring within one week. Rats infused on day five and day ten both hard mortality rates of 10%, and those injected thirty days after the myocardial infarction had a mortality rate of 30%, usually at three weeks post infarction. Before injection, all rats had a similar left ventricular ejection fraction of about 45%. After four weeks, the left ventricular ejection fraction in five-day transplantation group had moved up to about 50% and the ten-day group reached almost 60%, doing significantly better than the control groups. Left ventricle wall thickening also improved significantly in the 10-day group, as opposed to the others. While scar formation was extremely noticeable in hearts injected with buffered solution, scar size was smaller in groups that received the HUCBC injection, more so in the five and ten day groups than the others. When injected cells were immunostained with anti-human leukocyte antigen, it was observed that only cells delivered in the ten-day group had settled in the infarct zone in the myocardium enough to make a difference. New angiogensis was due to the endothelial progenitors found in HUCBC. Microvasculature density was significantly greater in the five-day and ten-day groups than in their controls, and was altogether greater in the ten-day group. Myocardial infarction is a time dependent process, based on the loss of effective cardiomyocytes and the formation of scar tissue. The process begins with an inflammation of the infarct zone, and this reaction is nearly complete at ten days post myocardial infarction. It can be concluded that this is the best environment for stem cell homing and survival (Xing, Yun-li et al. 2009).

Tissue Engineering

Cardiac muscle tissue has a very quick and active metabolism. It therefore requires a lot of oxygen and nutrition. In natural cardiac tissue, almost every cardiomyocyte can be found in close proximity to a capillary vessel, ensuring that each cell is properly perfused and taken care of. Cardiac cell density is much higher than most other tissues. In a normal heart, non-cardiomyocyte cells such as vascular endothelial cells, smooth muscle cells, and fibroblasts make up 70% of the tissue. These non-cardiac cells are integral to normal heart functioning. All these factors together make it very difficult to artificially create cardiac muscle (Guo et al. 2009).

One study investigated the effects of co-seeding rat mesenchymal stem cells with embryonic cardiomyocytes. The scientists conducting the study believed that the only way to attempt to engineer cardiac muscle is in three dimensions, so they constructed a three dimensional tubular scaffold called a myotubule. They fed liquid collagen between two rotating

cones, resulting in a cylindrical tube of collagen fibrils with a hollow, inner central lumen. To isolate the mesenchymal stem cells, they anesthetized 300 rats and removed the femoral and tibial bones. The marrow cavities were flushed and the marrow was combined. The bone marrow was passed through thin needles to break up clumps, and the single cell suspensions that resulted were centrifuged for five minutes at 200g. Cells were trypsinized three times, and replated. Only bone marrow stromal cells remained. Cells were labeled with green fluorescent protein for lineage tracing, and were replated for subculturing. Embryonic cardiac myocytes were collected from rat embryos. Upon the dissection of their hearts, the atria were discarded. The ventricles were minced and incubated, and centrifuged, until ventricular primary cells could be isolated. The embryonic ventricular cardiomyocytes (ECMs) were planted in a medium filled collagen tube alone, and a combination of ECMs were seeded together with mesenchymal stem cells. The results of both experiments were studied at seven, fourteen, twenty one, and twenty eight days. Gene expression of the tissue in both myotubules was studied in order to analyze cardiogenic differentiation. In order to validate findings of cardiac myocyte markers in these collagen tube cultures, cardiac specific markers were immunocytochemically stained. This was done using antibodies directed against specific cardiac transcription factors, hormones, contractile and structural filaments, and junctional proteins. Through staining and lasers, it was established that the cells located in the myotubes tested positive for many cardiomyocyte markers, including myosin heavy chains, sarcomeric myosin heavy chains (a contractile protein), cardiac troponin, cardiac actinin and desmin, GATA binding proteins, peptide hormones ANP and BNP, and others (Valarmathi et al. 2010).

At 21 days, ECM tube cultures showed the presence of myocytes mainly on the luminal and outer surfaces of the construct. External to the tubule, ECMs aligned and overlapped in an orderly manner, but cells on the inner luminal surface showed cord like cellular arrangement that resembles that of in-vivo myocytes. These cells showed signs of developing sarcomeric units, and tested positive for actin and myosin. They indicated progressive differentiation towards in-vivo neonatal-like ventricular cardiac muscle cells. In MSC/ ECM co-cultures, the differentiating cells appeared organized into many layers of intercalated bundles and branches throughout the myotubule. Cross bridges and specialized cell junctions were evident. The maturing cardiomyocytes showed evidence of evolving into Z-disks and into cardiac specific sarcomeric arrangements, and showed promise for cardiac biosynthetic activities. The MSCs that had been immunostained showed markers associated with cardiomyocytes, including myosin heavy chains and other aforementioned characteristic, proving that it wasn't only the ECMs affecting the expression of cardiac properties. In comparison to the ECM only construct, hormone secretion levels of the MSCs/ECMs stayed strong and constant. The cells were metabolically active. When tested under electron microscope, the nuclei of ECMs were typical of underdeveloped embryonic cardiac muscle cells. The MSCs/ECMs co-culture revealed the typical appearance of developing cardiomyocytes. Cells were elongated, multilayered, and orderly. Myofilaments were present, if randomly dispersed throughout. Plus, the cells showed developing mitochondria and vescicles of the active cardiomyocyte. MSCs/ECMs combination expressed cardiac specific genes, proteins, ion channels, receptors. In addition spontaneous, synchronized contraction of the culture was evident through the transparent myotubule (Valarmathi et al. 2010).

Previous successes in the implantation of engineered cardiac tissue have failed due to necrosis at the core of the transplanted tissue and poor survival related to ischemic injury.

Bioengineers have long sought a solution to this problem. Human embryonic stem cells were differentiated into cardiomyocytes and suspended in a rotating orbital shaker, resulting in human cardiac tissue patches, composed of enriched cardiomyocytes. These patches, however, did not survive transplantation in-vivo. Scientists tried heat shocking the patches a day before implantation and bathing them in prosurvival cocktail before implanting them into skeletal muscle of nude rats. At one week, they found only rare, isolated human cardiac muscle cells. The peripheral edges of the tissue were viable, but the entire inner core was dead. They deduced that the problem was in large part due to the ischemic injury at the infarct zone, and that the patches were too thick for nutrients to diffuse into the core, and that in order for the procedure to be successful, the tissue needed to be vascularized. The tissue patches died before host angiogenesis could provide them with a normal blood supply (Stevens et al. 2009).

Tri-cell cardiac patches were scientists' next attempt. Human embryonic stem cell derived cardiomyocytes were combined with human umbilical vein endothelial cells (HUVEC) and mouse embryonic fibroblasts in a 1:1:0.5 ratio in a medium of human embryoid bodies (a cluster of heterogeneous embryonic stem cells that are set to differentiate to a specific lineage [Itskovitz-Eldor et al. 1999]). The newly created tissue had endothelial cell networks that resembled a vascular plexus. The next logical step was to test the contractility of the new tissue. Patches were stimulated using square waves of frequency and contraction was monitored via video edge detection. Cardio-HUVEC-MEF patches were cultured for 2-3 days before testing. They routinely contracted when stimulated by 2 Hz of electricity (120 beats/minute), but couldn't keep pace with 5 Hz of stimulation. When stimulated at higher frequencies, the patches never fully relaxed, and a decrease in contractile ability resulted. Another important consideration for scientists to take into account are the passive mechanical properties of cardiac tissue, especially in relation to diastolic filling. The passive stiffness of Cardio-HUVEC-MEF patches were tested in comparison to cardio only patches by using strips cut from each patch. They were stretched in increasing length increments. At 7.9 mN/mm^2, cardio-HUVEC-MEF constructs were closer to the stiffness of neonatal pig myocardium than cardio-only patches. When tested, scientists saw that the cardio-HUVEC-MEF constructs produced more connective tissue and collagen fibrils per area, making their stiffness more physiologically appropriate. These patches were a thousand times less stiff than the collagen scar that forms as a result of myocardial infarction, suggesting that they would impede much less on diastolic filling (Stevens et al 2009).

The next step for scientists was to test whether or not these vascularized human cardiac tissue patches could survive implantion in vivo. Prior to their implantation into the gluteus muscle of nude rats, cardio-only and cardio-HUVEC-MEF patches were heat shocked and bathed in pro-survival cocktail. At one week post implantation, the rats were killed. Human cardiomyocytes and endothelial cells were indentified through the immunohistochemical staining of β-myosin heavy chain and human complement proteins. In cardio-only patches, only the occasional, isolated human cardiomyocytes were detected. Comparably, cardio-HUVEC-MEF patches formed much larger grafts of human myocardial tissue. The cardiac muscle cells showed small, sarcomeric arrangements. Many human endothelial cells could be found among the patches, some of them even containing traces of red blood cells. Despite the fact that cardio-only patches were produced from 50% more cardiomyocytes, implanted cardio-HUVEC-MEF patches grew to be 11 times larger. Next, the cardio-HUVEC-MEF constructs were sutured into the hearts of nude rats. The patches had attached to the hearts of all test subjects. The microvessels

that had formed contained red blood cells and white blood cells, indicated that the new vessels had anastomosed with the normal rat blood vessels, creating a much more ideal environment for tissue survival. (Stevens et al. 2009).

In the ten years that scientists have been experimenting with tissue engineering, much progress had been made. Nevertheless, there are still many obstacles that need to be overcome before newly synthesized cardiac tissue can be used to alleviate the effects of myocardial infarction. One of the largest problems facing bioengineers is constructing tissue the proper size and shape to fit into human myocardium. So far, no three dimensional tissue construct has been thick enough or large enough. Three to four cardiomyocyte monolayers have been successfully stacked one on top of the other, but the thickness hasn't come close to that of human cardiac tissue. As for shape, researchers have been able to construct tissue in rings, strips, and squares. While each of those shapes has its own advantage, none of them come close to the shape of a natural human heart. Of course, another large issue is immunorejection. None of the methods developed thus far have used or yielded autologus material. All implantation studies have used immunosuppressants. Another commonly overlooked problem is that most media supplements used to grow the tissue are xenogenic, resulting in several issues including infection. New, non-xenogenic growth media must be developed before engineered tissue can successfully be transplanted into humans (Xing, Yu-jie et al. 2009 b). Until this tissue has been successfully transplanted, studies cannot be done to test how this tissue affects the functionality of the working heart.

Conclusion

Because myocardial infarction and the death of cardiac muscle has always been seen as irreversible, researchers have been working on many different ways to reverse the process and regenerate cardiac muscle. In most studies, heart function was partially, if not mostly restored, even if cardiac muscle itself was not actually produced. The reperfusion of existing muscle and the prevention of scar formation definitely played a part in reestablishing cardiac function and preventing further heart complications. Researchers discovered the existence of cardiac progenitor cells and have begun to experiment with them. Tissue has been engineered and successfully transplanted into rats. Much progress has been made in the field, though there are still obstacles that need to be overcome. Many studies featured embryonic cells, the use of which is still extremely controversial, and may lead to tumor formation in the long run (Joggerst and Hatzopoulos 2009). Finally, more human trials need to be done to prove the safety and benefits of cell therapy and tissue transplantation.

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