CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THESIS

Adapted from "Young at heart. An update on cardiac regeneration."

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Minerva Med. 2010 Aug, 101 (4). 255-70

BACKGROUND

Ischemic heart disease is the leading cause of morbidity and mortality in industrialized countries.[1] A major contributor to ischemic heart disease is myocardial infarction (MI). The erosion or rupture of a coronary atherosclerotic plaque can result in the acute obstruction of myocardial blood supply leading to massive loss of cardiomyocytes. Since mammals are unable to replenish the large number of cardiomyocytes lost as a result of MI, a fibrotic scar with a definitive nature is formed.[2] Although the scar increases the tensile strength of the infarcted myocardium and thereby prevents cardiac rupture, it impairs the contractile capacity of the heart which will eventually culminate in heart failure. Important advances have been made in prevention and treatment of the acute complications of MI and in reducing myocardial infarct size by reperfusion strategies.[3] As a result, the number of patients in developed countries that acutely dies from MI has decreased in recent years. However, patients that do survive are prone to undergo (left) ventricular remodeling and to ultimately develop heart failure. Prognosis of advanced heart failure is only 50% survival after two years. The only therapy for heart failure that addresses the fundamental problem of cardiomyocyte loss and vasculature damage is heart transplantation. Unfortunately, this therapy is restricted since the demand for donor hearts is largely exceeded by donor availability. Furthermore, immune rejection of the transplanted heart remains a major problem. If one could reconstruct the myocardium by replenishing lost cardiomyocytes and blood vessels through cell-based therapy, this would provide a powerful approach to treat cardiovascular disease. The ideal cell population for cardiac cell therapy should be able to generate all major cell types in the heart including endothelial cells, pericytes and smooth muscle cells to form new blood vessels. Newly formed cardiomyocytes must integrate structurally, mechanically, electrically and metabolically with the host myocardium and beat synchronously to be of functional significance and to prevent arrhythmias. The field of cardiac regeneration was fueled by a study of Orlic and colleagues, who reported on the existence of a population of $Lin^{-}c\text{-}Kit^{+}$ bone marrow cells that appeared to cause infarct healing and partial restoration of cardiac function after injection into the border zone of freshly infarcted mouse hearts.[4] (As only 44±10% [n=6] of the donor cells were eGFP-positive). Histology analyses revealed the expression by the engrafted donor cells of cardiomyocyte, smooth muscle or endothelial markers and showed that the cells in the regenerated areas were mainly of donor origin. Based on the findings, the authors concluded that the damaged myocardium produces signals causing the donor cells to differentiate into cardiomyocytes, smooth muscle cells or endothelial cells instead of into hematopoietic cells However, in subsequent years, these spectacular results could not be confirmed.[5,6] Nevertheless, the use of bone marrow cells to reduce

the loss of contractile tissue following acute MI was translated to the clinic with an unprecedented speed. The results from placebo-controlled randomized trials at 5-year follow-up show that the administration of bone marrow-derived cells is safe[7] and on average has a very similar small positive effect on cardiac function as existing pharmacotherapies for heart failure. While initially the focus in cardiac cell therapy was on whole bone marrow or hematopoietic/endothelial subfractions of these cells, recently the attention has been largely directed to mesenchymal stem cells (MSCs; see below) and cardiac progenitor cells.

MESENCHYMAL STEM CELLS

CHARACTERIZING MSCS

MSCs are a source of cells that has received a lot of attention with regard to cardiac stem cell therapy due to their abundance and relatively easy isolation and handling. These cells were first characterized by Friendenstein et al., who described fibroblast-like cells derived from adult bone marrow.[8] As no unique marker has been identified yet to distinguish MSCs from other cell types, the International Society for Cell Therapy has put forward a set of criteria for defining human MSCs. These criteria comprise (1) the ability to adhere to tissue culture plastic under standard culture conditions, (2) the presence of a set of surface markers (MSCs have been described to be positive for CD73, CD90 and CD105, but do not express CD79a, CD45, CD34, CD31, CD19, CD14 or CD11b or HLA-DR on their surface as determined by fluorescence-activated cell sorter analysis), and (3) the capacity to differentiate in vitro into osteoblasts, adipocytes and chondroblasts.[9] Additionally, MSCs have been shown to secrete pro-angiogenic and anti-apoptotic cytokines and possess immunomodulatory properties.[10,11] Several studies propose that MSCs have cardiomyogenic properties, together with their pro-angiogenic and anti-apoptotic capacities, these features have made these cells a promising therapeutic tool for cell-based cardiovascular repair.

ORIGIN OF MSCS

Since their first identification, MSCs have been demonstrated in a wide variety of adult, neonatal, fetal, and also embryonic tissues.[12,13] However, differences exist between MSCs derived from various sources, but also from the same source and even from the same tissue isolation, which may have important clinical implications.[14] MSCs were originally isolated from adult bone marrow, but their numbers in this tissue are low.[8] It has been estimated that about 0.001% to 0.01% of cells in a bone marrow aspirate is able to attach and grow as fibroblast-like cells.[10] Recently, cells with similar properties as adult bone marrow MSCs have been found in adult

adipose tissue, albeit in higher numbers.[13,15] The large quantities of MSCs that can be derived from fat aspirates constitute an advantage over bone marrow-derived MSCs.[13,16] MSCs have also been isolated from other adult tissue sources such as lymphoid organs (thymus and spleen)[17], periodontal ligament[18], and peripheral blood[19], but these MSCs have not been used in clinical trials in contrast to adult bone marrow- and adipose tissue-derived MSCs. Recent studies have shown that the number and function of stem cells are depressed in older patients.[20,21] Moreover, regardless of age, stem and progenitor cell number and function are impaired in patients suffering from various cardiovascular risk factors, such as diabetes, hypercholesterolemia and hypertension.[20] Using younger, and likely more healthy sources may circumvent the limitations of adult stem cell therapy. The advantages of MSCs derived from young sources have been extensively described for cells derived from fetal and neonatal tissues, such as umbilical cord[22] and blood[23], amniotic fluid[24] and membrane[25], bone marrow, lung and liver.[26] These "young" MSCs show better intrinsic homing and engraftment, greater multipotentiality, increased ability to self renew and lower immunogenicity.[25,27] The use of young MSCs for cell therapy typically excludes autologous transplantation. Currently the efficacy of allogeneic strategies are being explored in animal models and in clinical trials to overcome this drawback.[28-30] Strategies to modify the immunogenicity of allogeneic MSCs are also being investigated to improve their potential in tissue repair.[31] Recently, MSCs have been derived from human embryonic stem cells (ESCs). These fibroblast-like cells were obtained after co-culture of the human ESCs with the OP9 murine bone marrow stromal cell line or directly from ESCs cultured without feeder cells.[32,33] They resemble MSCs from various other tissue sources with respect to morphology, surface marker profile, immunogenicity and differentiation potential toward osteogenic, adipogenic and chrondrogenic lineages. Moreover, they lack expression of the pluripotency-associated markers and after transplantation they do not form teratomas.[32-35] Although ESC-derived MSCs seem superior to other sources with regard to their differentiation potential, clinical use of embryonic stem cells is still surrounded by ethical issues. This makes it difficult to envision the clinical application of ESC-derived MSCs in the near future.

As MSCs have been shown to differentiate towards a cardiomyocyte-like phenotype, the question arises whether MSCs are involved in cardiac development. The group of Liechty et al. has shown that human MSCs engrafted and underwent site-specific differentiation into various cell types including cardiomyocytes after in utero transplantation in a sheep model.[29] Identical results were obtained by Mackenzie et al., who used a similar ovine model and was also able to identify cardiomyocytes of human origin.[36] These studies suggest that transplanted MSCs could participate in organogenesis during fetal development. Nonetheless, the heterogeneity of MSC populations obtained by plastic adherence and the wide variety of cell

surface markers used to isolate MSCs, make identification of a MSC population in the heart quite difficult.

IN VITRO CARDIAC DIFFERENTIATION OF MSCS

The ability of adult human MSCs to differentiate into functional cardiomyocytes remains a much investigated and debated topic. This may be due to application of different criteria to identify adult MSC-derived cardiomyocytes. While some groups report expression of cardiac sarcomeric protein genes, organization of the encoded proteins into sarcomeres is often absent. Also intrinsic action potentials are rarely detected in these cells indicating that while a cardiomyocyte-like phenotype may be present, a functional cardiomyocyte has not been formed.[37-39] In recent years, several groups have shown that MSCs derived from neonatal tissues can be induced to express cardiac proteins in a sarcomeric pattern while they also could generate an intrinsic action potential which suggests that young MSCs do have the ability to become a functional cardiomyocyte.[25,40] However, these studies do not include the use of species-, strain- or gender-specific markers in combination with cardiomyocyte markers to unambiguously demonstrate that the cardiomyocyte is indeed derived from a stem cell. Without these specific markers heterocellular fusion as a mechanism in the formation of MSC-derived cardiomyocytes can not be excluded. Strikingly, many studies showing both expression of cardiac muscle genes and generation of intrinsic action potentials in MSC-derived cardiomyocytes involve co-cultures of MSCs and native cardiomyocytes to induce cardiomyogenesis. This suggests that interactions with bona fide cardiomyocytes might play an important role in the induction of cardiomyogenic differentiation of human MSCs. With regard to cardiovascular regeneration, the potential of MSCs to differentiate into endothelial and smooth muscle cells is also important. Tamama et al. have shown that bone marrow-derived human MSCs can gain molecular but also functional characteristics of smooth muscle cells after treatment with a small molecular mitogen-activated protein kinase kinase inhibitor.[41] Inducing endothelial differentiation in MSCs has given inconsistent results. After exposing adult bone marrow-derived human MSCs to endothelial cell differentiation-inducing conditions for up to 12 days, the MSCs were able to form capillary-like structures on Matrigel.[42,43] A study by Roobrouck et al. also showed that human MSCs are able to differentiate into endothelial cells in the presence of vascular endothelial growth factor.[44] The aforementioned results are, however, were not in line with those of Au et al. and Delorme et al. who showed that bone marrow-derived human MSCs are not able to differentiate toward the endothelial lineage even after priming the cells under endothelial conditions.[45,46]

IN VIVO CARDIAC DIFFERENTIATION OF MSCS

Multiple in vivo studies have been conducted investigating the regenerative capacity of MSCs in the diseased heart.[25,47-50] Nevertheless, the ability of MSCs to undergo cardiac differentiation in vivo remains a highly controversial topic. While some studies showed expression of cardiomyocyte proteins in a sarcomeric organization after cardiac stem cell transplantation in an animal model, they did not use an additional marker to determine whether these cells were indeed of MSC origin (e.g. species-, strain- or gender-specific markers).[25,50] Also, Toma et al. did not look into the possibility of heterologous cell fusion as an explanation for the expression of cardiomyocyte proteins by the transplanted MSCs.[50] In contrast, Quevedo et al. showed that male MSCs are able to differentiate into cardiomyocytes, smooth muscle cells and endothelial cells after allogeneic stem cell transplantation in the chronically infarcted myocardium of a female swine by co-localization of the Y chromosome with markers of cardiac muscle, vascular muscle and endothelial lineages.[49] Nonetheless, in all the vivo studies mentioned above, the frequency with which MSCs differentiated into cardiac cells was low.[49,50] Furthermore, MSC engraftment rate in these studies is also minimal. Therefore, modest improvements in cardiac function observed in most animal studies have been ascribed to paracrine mechanisms. MSCs secrete cytokines and growth factors that can inhibit apoptosis and fibrosis, suppress the immune system and induce angiogenesis.[48,51-53] In recent animal studies, genetically modified MSCs have been used to increase their therapeutic efficacy. For example, overexpression of chemokine receptors by MSCs have been shown to improve cell viability, migration, engraftment and capillary density in the injured myocardium.[54] Furthermore, paracrine factors that are released by MSCs that overexpress GATA4 increase angiogenesis and cell survival.[55] Also, Akt overexpression led to repair of the infarcted myocardium and improvement of the cardiac function.[56] Therefore, genetic modification of MSCs may be a tool to increase their effectiveness in mediating cardiac repair.

CLINICAL TRIALS OF MSC THERAPY FOR CARDIAC REPAIR

Clinical cardiac stem cell therapy for acute MI and ischemic cardiomyopathy using bone marrow-derived MSCs has been conducted.[28,57,58] In these phase I/II trials different cell delivery routes, such as intravenous and intracoronary infusion and intramyocardial injection were investigated. The main objective of these studies was determining safety of both autologous and allogeneic MSC therapy. Chen et al. investigated the effects of intracoronary autologous MSC infusion in patients with acute MI. Compared to controls, which were infused with saline, cardiac function

was modestly improved in patients who received MSCs. More important, however, was that this study showed intracoronary autologous MSC infusions to be safe with regard to adverse events such as occurrence of arrhythmias after cell administration. Moreover, no deaths were reported during the 6-month follow-up period.[57] Hare et al. investigated the safety and efficacy of intravenous allogeneic human MSC infusion in patients with acute MI. This randomized, double-blind, placebo-controlled dose-escalation study showed that allogeneic intravenous MSC delivery is safe in acute MI patients and also improves cardiac function in these patients compared to that in controls.[28] This study is especially interesting as multiple studies have shown that functional capacities of stem cells decline with age.[59,60] Use of allogeneic stem cells from young donors may help to increase the therapeutic effect of cardiac cell-based therapy.

Cardiac MSC therapy has also been initiated for patients with cardiac injury post-MI. Williams et al. showed that intramyocardial injection of autologous bone marrow-derived MSCs in patients with ischemic cardiomyopathy improved regional contractility of a chronic myocardial scar and led to reverse remodeling. Importantly, the intramyocardial cell injections did not cause sustained ventricular arrhythmias. This clinical trial shows that cardiac stem cell therapy in patients with ischemic cardiomyopathy is safe and has positive effects on cardiac structure and function.[58] The findings of this early-phase clinical trial have led to larger studies in which bone marrow-derived MSCs will be compared to mononuclear cells (TAC-HFT trial)[61], autologous MSC therapy will be compared to allogeneic MSC therapy (POSEIDON study), MSCs will be delivered during coronary artery bypass surgery (PROMETHEUS trial) and MSC therapy will be investigated for treatment of idiopathic dilated cardiomyopathy (POSEIDON-DCM study). In addition, another clinical trial in which MSCs are primed ex vivo with cytokines to improve cardiac differentiation in vivo has been initiated.[62] While the smaller clinical studies have shown that stem cell therapy is safe and feasible and modest improvements in cardiac function were achieved, the outcomes of the larger double-blind, randomized-controlled clinical trials will have to reveal whether MSC-based cardiac therapy is truly beneficial.

The current experience with the use of MSCs to treat cardiac diseases has provided several leads to improve their therapeutic efficacy. First of all, the option of using allogeneic stem cells from young donors in cardiac stem cell therapy should be thoroughly investigated as several studies have exposed that the functional capacity of stem cells declines with age.[59,60] Also, another major issue regarding cell transplantation that deserves examination is the low survival and engraftment rate that have been reported in studies in which MSCs were injected.[25,48,50] Recent studies have focused on improving engraftment rate by using biomaterials.[63-65] The risks of adverse events (e.g. occurrence of arrhythmias) that could occur when the

engraftment rate is improved are largely unknown and future studies should also take these into account. Besides engraftment, alignment with the surrounding myocardial tissue also seems to be of importance as alignment of stem cells could influence the mechanical and electrical activation of the heart. Currently, cardiac patches have been engineered to improve structural and electrical integration in host myocardium after cardiac stem cell transplantation.[66-68] Before MSC-based therapy will find widespread application in the clinic, the issues raised above should be answered in extensive in vitro studies, animal experiments and clinical studies.

AIM AND OUTLINE OF THE THESIS

In recent years stem cell based therapies have shown to give modest improvements in heart function. Most clinical trials have been conducted with autologous and therefore adult stem cells. However, functional capacity of stem cells decline with age. Therefore, **chapter II** of this thesis focuses on the influence of donor age on the in vitro differentiation potential of mesenchymal stem cells (MSCs) towards three cardiac lineages, namely cardiomyocytes, smooth muscle cells and endothelial cells. The exact mechanism behind improvement of cardiac function after stem cell transplantation is unknown, but functional integration with host cardiac tissue is known to be important for therapeutic efficiency and to avoid adverse effects.[69] The myocardium has a typical anisotropic tissue structure, which affects electrical and mechanical activation. Therefore, MSC-derived cardiac cells should align properly with native cardiac cells in order to restore tissue structure and for anisotropic conduction. **Chapter III** discusses the influence of forced alignment of MSCs undergoing cardiomyogenic differentiation on their functional integration with myocardial cells. Besides functional integration, electrical coupling with the surrounding host tissue is fundamental to make MSC-based therapy a safe option. Clinical trials in which skeletal myoblasts were transplanted in the diseased myocardium emphasized the importance of gap junctional coupling with the resident cardiomyocytes. Transplantation of skeletal myoblasts that after differentiation into myotubes do not eletromechanically couple with host myocardium led to an increased incidence of arrhythmias in patients.[70-72] Gap junctional coupling also seems to play an essential role in inducing cardiomyogenic differentiation. Multiple studies show stem cells are able to undergo cardiomyogenesis when they are in close contact with native cardiomyocytes. Therefore, **chapter IV** evaluates the role of gap junctional coupling in the cardiomyogenic differentiation potential of human MSCs. As cardiomyogenic differentiation of stem cells is often investigated after intramyocardial transplantation or in co-cultures with cardiomyocytes, they are commonly labeled through viral transduction with a marker protein to facili-

tate their identification. An often neglected pitfall of these studies is secondary transduction of cardiomyocytes by viral vector-marked stem cells and a second marker to distinguish stem cell-derived cardiomyoctes from native cardiomyocytes is rarely used. In **chapter V**, secondary transduction of neonatal rat cardiomyocytes by adult human cells that had previously been transduced with an enhanced green fluorescent protein-encoding lentiviral vector was studied.

The initial results of cell transplantation studies show improvement in cardiac function, however, therapeutic efficiency is not optimal due to low survival and engraftment rate of the transplanted cells. Biomaterials have been developed to improve survival and engraftment rate and thereby therapeutic efficiency. The possible adverse effects of a higher engraftment rate and of the pattern of distribution of a higher number of transplanted cells are unknown. These aspects are studied in **chapter VI**, which explores the role of engraftment patterns of MSCs on arrhythmicity in controlled *in vitro* models. Not only transplantation of exogenous cells in the diseased myocardium could lead to arrhythmias, proliferation of endogenous myofibroblasts after MI is known to cause conduction disturbances. An alternative way to minimize the negative effects of MI is described in **Chapter VII** of this thesis. In this chapter in a vitro model is used to study whether anti-proliferative treatment of myofibroblasts prevents arrhythmias by limitation of myofibroblast-induced depolarization.

Chapter VIII provides the summary and conclusions of this thesis, as well as future perspectives related to stem cell-based therapies for the treatment of damaged myocardium.

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