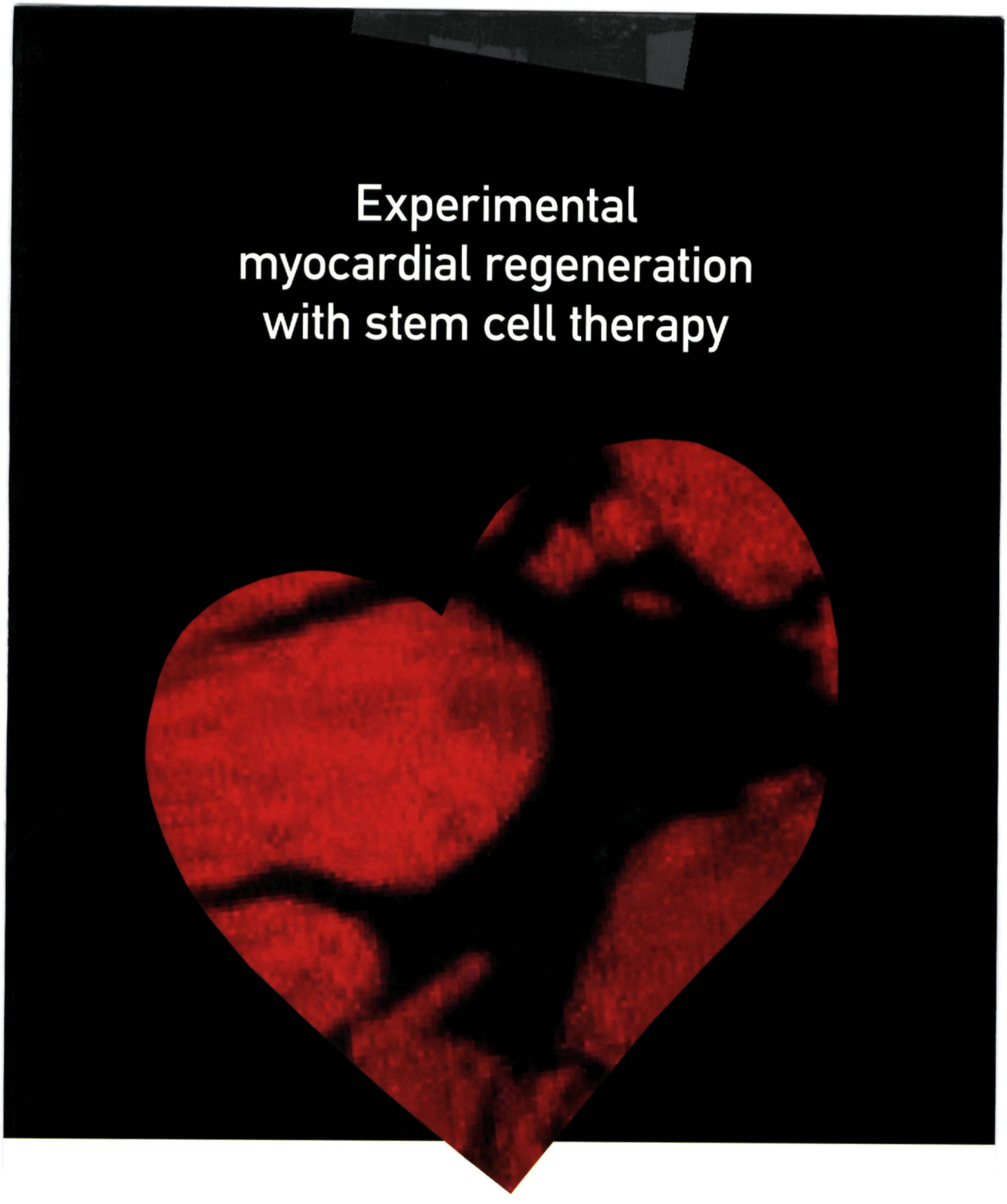
Experimental myocardial regeneration with stem cell therapy



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Experimental myocardial regeneration with stem cell therapy

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Experimental myocardial regeneration with stem cell therapy

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Voor mijn ouders

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CHAPTER 1

General Introduction

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and
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Part of this chapter is based on:
From mice to men: healing the heart with stem cells;
provisionally accepted by Stem Cell Reviews and Reports

ABSTRACT

There is a growing interest in cell therapy as a new treatment of myocardial infarction and heart failure. In this review we will discuss the current results of cell therapy studies and consider the possibility to replace damaged myocardium with functional tissue. Different types of stem cells have been extensively studied, including embryonic stem cells, bone marrow-derived stem cells and skeletal myoblasts. Interestingly, researchers also found the heart to possess stem cells that can be used to regenerate the damaged heart. Several delivery routes to administer the cells have been examined and they all show beneficial effects; however, there are some limitations. Intramyocardial injection has resulted in arrhythmias, and intravenous delivery of stem cells remains controversial since the cells can home to all organs with the risk of developing neoplasms. Since different mechanisms such as transdifferentiation, cell fusion, release of growth factors, neovascularization and decreasing apoptosis have been suggested to explain the positive effects of cell therapy, we conclude that it is most likely multifactorial.

INTRODUCTION

Myocardial ischemia and acute myocardial infarction (MI) are the leading causes of heart failure in Western society and major causes of morbidity and mortality throughout the world [1,2]. It is well known that extended periods of myocardial ischemia cause tissue injury and cell death. The standard belief that the heart does not have an endogenous regenerative capacity to make up for these lost cells and contains myocytes that are terminally differentiated has been questioned. There have been studies that prove myocyte proliferation exists and, furthermore, undifferentiated cells have been found in transplanted human hearts [3,4]. However, the proliferation is never sufficient to compensate for the millions of cardiomyocytes that are lost after acute MI [5]. And without early reperfusion of the infarct-related artery, which is not always possible, this results in necrosis, scar formation, left ventricular remodeling and mortality [6,7]. Once the infarct has occurred, present treatment is based upon prevention of additional deterioration of the myocardium. This is found in a combination of drug therapy, percutaneous coronary intervention, coronary bypass grafting, device therapy and as a final option heart transplantation [8].

However, these therapies do not repair any damaged myocardium, they merely have a preventive function. Thus far, the only therapy that has proven to diminish fibrosis is stem cell therapy. The lost myocytes can be replaced by stem cells and restore damaged myocardial tissue with functional tissue and improve diminished contractile function [9-13]. Different cell

types, as well as methods of delivery, have been used to study the benefits of cell therapy in the heart. In our studies, we have predominantly used embryonic stem cells, because of their infinite proliferative and developmental capacity [14]. For a complete overview of regenerative therapy we will also discuss adult stem cells, skeletal myoblasts, fetal cardiomyocytes and endothelial progenitor cells. Different mechanisms have been proposed to explain the beneficial effects of cell-based therapy. These include cell transdifferentiation, cell fusion, release of growth factors, preventing cardiac apoptosis and neovascularization.

Cell types for myocardial regeneration

Every type of donor cells has its own advantages, limitations and practicability in certain pathological settings (Table 1).

Embryonic stem cells

Embryonic stem cells (ESCs) are very interesting to use in cell therapy studies, since they have the ability to develop into any possible cell type. This remarkable potential holds a promise for future therapies and was the main objective for utilizing these cells in our studies. In 1998, investigators were able, for the first time, to isolate cells from early human embryos and grow them in culture [15]. Day-5 blastocysts are used to derive ES cell cultures. To obtain ESCs, the trophectoderm has to be removed and the remaining inner cell mass (ICM) is then ready to be cultured [16]. The ICM cells are pluripotent, which means they are able to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm and possess the ability to generate any cell type of the body [16].

Human ES cells could be utilized in regenerative medicine and tissue replacement, to screen potential toxins and to acquire new methods for genetic engineering. After subjecting mice to an infarction and injecting them with stem cells, ESC-derived cardiac myocytes (CMCs) displayed spontaneous contractile activity, formed stable grafts and improved cardiac function significantly [17-19]. Furthermore, the CMCs derived from ESCs have been demonstrated to express several cardiac-specific markers [17-19]. Also, ESC-derived cardiomyocytes couple electromechanically to adjacent myocytes via gap junctions after being transplanted into the myocardium [17,20].

However, there are some downsides to the use of ESCs for cell therapy. The trouble with employing undifferentiated embryonic stem cells with an ability to form unlimited multiple cell lines is the risk of developing a teratoma [21,22]. For that reason, most stem cell transplantation studies worked with early-differentiated stem cells. Furthermore, the allogenic nature of ESCs gives concerns about immunological rejection [23,24] and the use of this type of cell gives rise to numerous ethical and legal discussions. Because of these discussions, the availability of human embryonic stem cells for research and therapy is limited.

Table 1. The advantages and limitations of different types of stem cells.

Stem cell types	Improve cardiac function?	Pros	Cons
Embryonic stem cells	Yes	Pluripotent Easily cultured	Teratoma Immunological rejection Ethical concerns
Adult stem cells	Yes	Autologous nature	Limited differentiation Limited cell numbers
Skeletal myoblasts	Yes	Easily cultured No ethical issues	No electromechanical coupling Low survival rate
Fetal cardiomyocytes	Yes	No arrhythmias	Immunological rejection Ethical issues
Adult cardiac myocytes	Yes	Autologous nature	Limited cell numbers
Endothelial progenitor cells	Yes	Autologous nature	Limited cell numbers

Adult stem cells

The history of research on adult stem cells began about 40 years ago, when researchers discovered that bone marrow contains at least two populations of cells: hematopoetic stem cells and bone marrow stromal cells. Since then, a great deal of research has been done, and adult stem cells have been documented in many organs and tissues, such as the blood stream, retina, liver, skin, gastrointestinal tract and pancreas [25,26]. The source of these stem cells is of great importance, since adult stem cells are generally limited to differentiate into cell types of their tissue of origin [26].

Bone marrow stromal cells (BMSCs) have been used in several studies of myocardial regeneration therapy. After stimulation, bone marrow stromal cells have been shown to express structures similar to cardiomyocytes and various cardiac specific proteins. These regenerated cardiomyocytes demonstrated spontaneous contraction and displayed sinus node and ventricular like potentials [11,27,28]. By expressing connexin 43, a gap junction protein responsible for intercellular coupling, adult stem cells have showed to be capable of electromechanical coupling with cardiomyocytes [11].

Furthermore, it has been demonstrated that intramyocardial injection of *lin* , *c-kit*$^+$ bone marrow cells in mice after infarction induced formation of new myocytes, endothelial cells and smooth muscle cells, hence restoring myocardial tissue [29] Studies with adult stem cells do not always demonstrate differentiation of stem cells into cardiomyocytes, suggesting different mechanisms of action contributing to the improved results [30] These mechanisms will be discussed in the latter part of this paper

An advantage of employing adult stem cells is that the patient's own stem cells could be used in the therapy, thus avoiding rejection of the immune system Also, BMSCs are an appealing option, since they enclose no ethical issues The drawback is that adult stem cells are rare and not easily grown in culture, while a large number of cells are needed for stem cell replacement therapies

Skeletal myoblasts

Skeletal myoblasts (satellite cells) are stem cells located at the basal lamina of the adult skeletal muscle and preserve the regenerative potential of the skeletal muscle [31] Using them for cell therapy requires harvesting skeletal muscle, expanding cells in vitro and re-injecting them into the patient There has been significant proof that skeletal myoblast transplantation improves cardiac performance in animals that suffered myocardial tissue injury [32-34] These studies have demonstrated that myoblasts can differentiate and develop into striated cells within the damaged myocardium and improve myocardial functional performance [32-34] Jain and colleagues studied the transplantation of skeletal myoblasts and found these cells to improve contractile function and systolic and diastolic regional function of the heart [35] The mechanism of this improved cardiac function remains unclear, one possibility is a combination of release of paracrine factors and improved angiogenesis Also, the newly formed muscle tissue in the infarcted area may change the compliance of the myocardial tissue, leading to less dilation of the ventricles [36] In addition, some of the myoblasts might become quiescent satellite cells that could participate in tissue repair during periods of ischemia By transplanting ischemia-resistant skeletal myoblasts, cell engraftment and survival in infarcted regions of the heart can be achieved more easily [37] Also, utilizing these cells avoids concerns as immunosuppression, the shortage of donor tissue and ethical dilemmas

Nonetheless, using myoblasts for cell therapy has its limitations There is a time delay between infarction and therapy, since there is a need to expand these cells in a laboratory However, the largest downside is the high risk of ventricular arrhythmias. No studies have shown electromechanical coupling between the transplanted myoblasts and the resident

myocytes, which is the probable cause of the reported arrhythmias [38,39]. Another well-known limitation is the low percentage of myoblast survival after transplantation [40].

Fetal cardiomyocytes

Fetal cardiomyocytes (FCMCs) have been extensively studied to replace lost CMCs with functional cells. Transplanting myocytes is a potential therapy form to increase LV function after MI, and Soonpa et al. [41] were the first to demonstrate transplanted fetal myocytes that could survive and form intercellular connections with host cardiomyocytes, beating synchronously and thus contributing to improved contractility. Other studies showed that implantation of FCMCs after myocardial ischemia increased the ejection fraction by increasing the density of capillaries and organizing their contractile proteins into sarcomeres and linking them together to form new cardiac tissue. The enhanced contractile function remained for at least 6 months [42-46]. Also, the engrafted fetal cells are a likely potential source of growth factors. These factors could promote neoangiogenesis in grafted areas through a paracrine effect. The increased microcirculation offers increased perfusion and could also purify the post-infarct necrotic area [47].

The main concern of using fetal cardiomyocytes is immunological rejection. Many studies showed that despite the use of cyclosporin, there was proof of lymphocyte infiltration [48]. In addition, there are still unresolved ethical issues as well as limitations in harvesting enough cells to repair damaged myocardium.

Adult cardiac stem cells

The adult heart has historically been considered to be a postmitotic organ. Until recently, the regenerative capacity of the heart has been questioned. However, current studies have shown isolated stem cell antigen-1 (sca-1^{+}, a stem cell marker) cardiac stem cells from murine hearts and the existence of cycling ventricular myocytes in the adult human heart [49, 50]. Also, cardiac and myocyte progenitors were found postmortem in pathologic human hearts [51]. These findings turn adult cardiac stem cells into yet another appealing cell type for cellular cardiomyoplasty. They are ideal because of their autologous and self-renewing origin and their minimal risk of arrhythmias. Cardiac stem cells have the ability to differentiate into myocytes, smooth muscle and endothelial vascular cells. These circulating stem cells can replace lost myocytes throughout adult life and are capable of regenerating part of the myocardium after significant myocyte loss [50,52-54]. Promoting the cells to home to injured myocardium could boost the repair. This has been tested by injecting growth factors in the myocardium of dogs after AMI. This resulted in stimulation of the cardiac stem cells to migrate and form new myocardium, which ultimately lead to improved postischemic

ventricular and myocardial functions [55]. Isolating cardiac stem cells from rats and reinjecting them in ischemic myocardium has demonstrated to regenerate ventricular wall, made up of new vessels and young myocytes, and improve left ventricular function [52]. However, there are still concerns regarding harvesting the cardiac stem cells and their multipotency that will need to be addressed in future studies.

Endothelial progenitor cells

Endothelial progenitor cells (EPCs) can be isolated from the bone marrow as well as the systemic circulation. Recently, EPCs were successfully isolated as CD34+ mononuclear cells and following studies have also discovered cell surface expression of CD133 and VEGF-2 [56-58]. EPCs respond to ischemia and the mobilized cells home to sites of neo-vascularization and differentiate into mature endothelial cells. The number of circulating EPCs correlates with the development of cardiovascular disease [59]. The role of EPCs in improving ischemic sites has triggered new approaches to cell therapy in ischemic diseases [60-62]. These cells could be ideal for cell therapy since they are autologous, which eliminates the need for immunosuppression.

Vascular trauma mobilizes EPCs in the circulation through the release of signal factors such as VEGF, granulocyte colony-stimulating factor (GCSF) and stromal cell-derived factor-1 (SDF-1) [63,64]. Intracoronary, as well as intravenous administration of granulocyte colony-stimulating factor after myocardial ischemia, mobilized endothelial progenitor cells and was associated with a significant increase in LVEF, enhanced neovascularization, limited apoptosis and consequently improved cardiac function [65]. Kawamoto et al. studied a model where human EPCs were transplanted in the hearts of rats after myocardial ischemia and showed these cells to differentiate into mature ECs, increase neovascularization and decrease myocardial fibrosis [66].

Intravenous transplantation of human EPCs causes them to migrate to ischemic sites. Thus, both cultured and isolated EPCs can have a beneficial effect [66,67]. The major obstacle to the clinical application of EPCs is the limited number of cells that can be obtained from the patient. For an average-size human being, 8-12 L of peripheral blood is needed to acquire the requisite number of cells. Expanding endothelial cells is possible, but may suppress their homing ability and limit their efficiency [60].

Methods of delivery

Successful delivery of stem cells to the injured myocardial region means the delivery of a sufficient number of stem cells to the damaged area and is a key factor to achieve cardiac repair. Also, the safety of the cell delivery is of great importance, as each approach has its

own complications and risks (Table 2). Common approaches of local cell delivery include intra-coronary, transepicardial and transendocardial transplantation and as a less invasive strategy the intravenous injection of stem cells.

Intracoronary injection

This delivery mode is especially attractive post-MI, when adhesion molecules and chemokines are upregulated and able to attract the stem cells to home to the infarcted and peri-infarcted area. Studies have shown that delivery of stem cells 4-9 days post-MI was most beneficial [68].

Stem cells can be injected into the coronary arteries by means of a percutaneous transluminal coronary catheter, in a similar manner as in coronary angioplasty. To enhance the time in which the stem cells can home, the coronary blood flow is stopped for a few minutes. The advantage of this type of delivery is that a maximum concentration of cells is able to migrate to and disperse in the infarcted areas where they can contribute to restoring cardiac function. The distribution of the stem cells increases electrical stability and reduces the risk of arrhythmias. Another benefit is that no surgical intervention is needed [53,69-72]. Even though cells are delivered directly in the infarcted myocardium, only 1-2% of the injected cells were identified in that area [73]. Another method of delivery into the coronaries is the trans-coronary venous injection, where a catheter is placed into the coronary sinus and cells are infused under high pressure. Besides its safety, this delivery mode offers a homogeneous distribution of cells. Using this method, skeletal myoblasts have been successfully delivered to damaged myocardium in patients with cardiomyopathy [74-76], the difficulty being the often irregular anatomy of the myocardial veins, which makes injection of the cells complicated.

Table 2. The advantages and risks of different cell delivery methods.

Method of delivery	Advantages	Limitations
Intracoronary injection	Even distribution Save method	Loss of cells
Intramyocardial transplantation	Precise delivery Less cells needed	Invasive method Arrhythmias
Systemic injection	Noninvasive delivery	Loss of cells Migration to other organs

Intramyocardial transplantation

Direct injection of stem cells is a worthy option when intracoronary injection is not, because of partial or total occlusion of the artery. Intramyocardial injection requires fewer cells to be administrated compared with intracoronary or intravenous infusion. However, intramyocardial injection is the most invasive delivery method, requiring open-heart surgery. Also, there is an increased risk of arrhythmias associated with this delivery method [77]. In a transepicardial approach, the cells are injected into the regions of the infarcted myocardium, while the region can be directly visualized. This is an ideal method, when an invasive procedure is already planned [78]. A transendocardial approach can be performed using a needle catheter across the aortic valve which is then placed against the endocardium where they can be injected into the left ventricle [79]. A useful instrument for this method is electrophysiological mapping, which can contribute to distinguish between viable and ischemic myocardium [80].

Systemic injection

Intravenous administration of stem cells is a convenient way of delivering cells to the heart. However, when the heart does not transmit homing signals, stem cells will not reach the desired destination, therefore being the greatest shortcoming of this method. Consequently, it is important that if embryonic stem cells are used, they have been partially differentiated in vitro before injection. Also, the stem cells should be injected within a few days of an acute MI, in order for the cells to home. Animal studies have proven that stem cells migrate to the heart after systemic injection and restore damaged myocardial tissue after acute MI or a myocarditis [81-83]. The addition of calcium to the stem cells is a way to enhance the speed and likelihood of migration of the transplanted cells. For the treatment of chronic heart diseases, a possibility is to first inject cytokines such as TNF-alpha, in order to increase the probability of stem cell homing to the heart [84,85].

Methods of regeneration

Researchers are still attempting to comprehend the ability of stem cells to play a part in repairing the heart. The theories that have been described include transdifferentiation, cell fusion, release of growth factors, preventing cardiac apoptosis and neovascularization. It is most probable that the beneficial effects that are monitored are caused by a combination of mechanisms.

Transdifferentiation

Transdifferentiation refers to the conversion of stem cells to another type of cell. In our studies regarding transplantation of embryonic stem cells in a mouse myocardial infarction model, we observed the differentiation of green fluorescent protein (GFP)-transfected stem cells into GFP-positive cardiac myocytes [19,82]. Many studies showed similar results, supplying evidence for this type of mechanism [10,13,20,32,34]. Transplanted ESCs could differentiate into three myocardial cell types: cardiomyocytes, vascular smooth muscle and endothelial cells [11]. The new cardiomyocytes expressed several cardiac specific factors, such as myosin, sarcomeric actin, connexin 43, GATA-4, Csx/Nkx2.5 and MEF-2 [52]. The common method for in vitro differentiation is through hanging drop and the formation of embryoid bodies in which beating cardiomyocytes are found [17,86-89]. The addition of VEGF and AZA-5 increases the amount of differentiated cells [88,89]. Culturing embryonic stem cells with endoderm-like cells resulted in the creation of cardiomyocytes as well [30]. However, there has been a lot of controversy surrounding this theory, as other studies used techniques to track cell fate and found none or a low number of the implanted stem cells actually differentiated [90,91].

Neovascularization

As a response to a prolonged period of ischemia, the body compensates by generating collaterals by either angiogenesis, arteriogenesis or vasculogenesis. Vasculogenesis is the formation of vascular structure from mostly endothelial progenitor cells from peripheral blood and growth factors such as bFGF and VEGF and could be a possible mechanism for the improvement of cardiac function after stem cell transplantation [65,92-94]. The endothelial cells needed for neovascularization may be generated by transdifferentiation of either transplanted or endogenous stem cells and/or by proliferation through secretion of angiogenic factors [95,96].

Decreasing apoptosis

After myocardial infarction and also in the failing heart [97,98], the number of apoptotic myocytes increases and stem cells could play an essential role in preventing this phenomenon. We demonstrated that stem cells are capable of replacing lost myocytes after myocardial ischemia. Transplantation of embryonic stem cells significantly reduced the number of apoptotic nuclei in the peri-infarcted region. Moreover, our in vitro studies showed that after being subjected to hypoxia, the number of apoptotic cardiomyocytes significantly decreased when cocultured with stem cells [99]. Our results are similar to other studies that showed that transplantation of stem cells protects against apoptosis by increasing neovascularization and

the induction of regeneration of endogenous cardiomyocytes [100-102]. The exact mechanism behind the anti-apoptotic effect of stem cells still needs more research; however, paracrine factors released from stem cells are most likely to play an important part in the process.

Releasing (growth) factors

Stem cell therapy may also be beneficial because of the effect they have by releasing growth factors and cytokines. Studies have shown that cell transplantation in ischemic muscle increases levels of growth factors, such as bFGF and SDF-1alpha [103,104]. Yoshioka et al. showed that grafted stem cells secrete VEGF, which is believed to increase myocardial blood flow [105]. Perfusion is also increased, which may be caused by a paracrine mechanism [106]. Injection of growth factors like VEGF, hepatocyte growth factor, insulin-like growth factor-1, stem cell factor, HGF and IGF-1 after myocardial ischemia, has demonstrated to enhance homing, proliferation, differentiation, survival of the transplanted stem cells, activation of native stem cells and thus resulted in myocardial repair and improved left ventricular functions [107-113]. Various studies have studied the effect of stem cell mobilization by granulocyte colony-stimulating factor, the latest meta-analysis of these studies showed no significant beneficial effect of G-CSF on cardiac function [114].

Fusion

Fusion means the transfer of cell contents from transplanted to host cells with the design to rescue the damaged host cells and might be important in the homeostasis of organs. Cell fusion may result in the rescue of damaged myocytes otherwise destined for apoptosis or necrosis, or produce hybrid cells that can release therapeutically active factors into the cardiac milieu [115-117]. There has been a lot of controversy surrounding cell fusion as a mechanism to improve cardiac function by using stem cells. Cell fusion with cardiomyocytes has shown to occur at a very low incidence, therefore making it unlikely for this mechanism to contribute to the significant effects that are achieved with stem cell transplantation [115]. In our studies we used the cre-lox recombination method to detect cell fusion events and found that fusion occurs in a greater number when the myocytes are hypertrophic [118]. Alvarez-Dolado et al. used the same method to demonstrate that after stem cell transplantation cell fusion caused the generation of myocytes and found evidence of spontaneous cell fusion in the heart [116].

Clinical trials

After the many results that were achieved with stem cells in animal studies, several clinical trials have been initiated. Menasche et al. used autologous skeletal myoblasts and transplanted them through an intramyocardial route during heart surgery. Cardiac function

increased significantly, but some of the patients also suffered from malignant ventricular arrhythmias and needed internal cardioverter defibrillators [119]. Several other studies have used autologous bone marrow cells and delivered these via intracoronary infusion, on average on the fourth-eighth day after myocardial ischemia. The Reinfusion of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) study showed positive results with this setup. There was a significant improvement in left ventricular function compared to the placebo group [120]. The Transplantation Of Progenitor Cells And REcovery of LV Function in patients with Chronic ischemic Heart Disease (TOPCARE-CHD) trial demonstrated only a modest improvement in heart function when bone marrow cells were used; however, none with circulating progenitor cells [121]. In contrast, the Autologous Stem cell Transplantation in Acute Myocardial Infarction (ASTAMI) trial showed no significant change in cardiac function after stem cell transplantation, compared to the placebo group [122]. However, even with negative results of this trial, and the many unanswered questions that need to be addressed, stem cell therapy is on its way to become a new and exiting form of therapy to cure heart diseases. A key result in the studies was the demonstration of viability and safety of stem cell therapy, which opens the road for many trials to follow.

Aim and outline of the thesis

In the field of cardiology, stem cell therapy has gained a position as potential new form of treatment. With millions of myocytes lost in the process of aging and after myocardial infarction, cell therapy might be an answer to this problem as stem cells have the remarkable capacity to restore and repair cells in several organs, such as the heart. This introduction describes the several studies that have been done to determine the best strategies to use stem cells in a clinical setting. We propose that not one but different mechanisms could be important for the beneficial effects of cell-based therapy and embryonic stem cells are the best type of cells to study cell therapy, because of their infinite proliferative and developmental capacity. The overall aim of this thesis is to achieve effective, therapeutically relevant and save stem cell delivery to the heart. The specific aims of this thesis are to:

1. Assess the efficacy of stem cell therapy to generate healthy heart muscle after myocardial infarction but also in the aging heart and in the heart suffered from myocarditis.
2. Determine which mechanisms explain the positive effect stem cell therapy has in the treatment of heart diseases.
3. Investigate the mechanisms of stem cell homing in order to achieve succesfull delivery of the cells to the hearts.

In order to achieve these research goals we first studied the properties, behaviour and migratory patterns of murine embryonic stem cells under different conditions. In **Chapter 2** we investigated the effect of cytokines and second messengers on the homing of embryonic stem cells and their ability to form different membrane extensions under different conditions. **Chapter 3** continues to describe that cardiac inflammation and the release of cytokines is important for the homing abilities of stem cells. We investigated this in a murine model of myocarditis, in which the stem cells were injected intravenously by tail vein and cardiac function was evaluated before and after stem cell transplantation to determine the succes of stem cell homing. **Chapter 4** describes experiments designed to evaluate the effect of intravenously infused embryonic stem cell-derived cells on cardiac function and regional blood flow in a myocardial infarction model. The proposed mechanism in this study that contributes to the enhancement of cardiac function is transdifferentiation. In **Chapter 5** we hypothesized that cardiac function in the setting of myocardial infarction might be improved after stem cell transplantion by preventing apoptosis of cardiomyocytes. Since aging is associated with loss of cardiomyocytes due to apoptosis, we investigated the anti-apoptotic effect of stem cells by using aging rats. **Chapter 6** describes another mechanism which might contribute to a benefical effect on stem cell therapy; cell fusion. Cell fusion appears to occur in a very low incidence under normal conditions and in our studies we aimed on studying which agents could promote fusion and advance stem cell therapy. In **Chapter 7** we investigated if engrafted embryonic stem cells could improve myocardial function in aging hearts and evaluated if this was linked to an increase in myocyte numbers and enhanced blood perfusion. In **Chapter 8** we discuss the main findings of the studies presented in this thesis and propose future plans.

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CHAPTER 2

Calcium and cyclic nucleotides affect TNF-α-induced stem cell migration

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ABSTRACT

The purpose of this study was to study the effect of calcium, cyclic AMP (cAMP) and cyclic GMP (cGMP) on embryonic stem cell (ESC) motility during TNF-α-induced chemotaxis. ESCs were monitored using a chemotaxis chamber, with different concentrations of calcium or cAMP or cGMP added to the medium. Changes in intracellular calcium ($[Ca^{2+}]_i$) were measured with the fluorescent dye fura-2/AM. We combined migratory parameters in a mathematical model and described it as 'mobility'. After adding calcium, a dose-dependant increase in cell speed was found. Cyclic AMP increased mobility as well as the $[Ca^{2+}]_i$. In contrast, adding dbcGMP resulted in a significant decrease in the mobility of the ESCs. During migration ESCs showed an increase in $[Ca^{2+}]_i$. Furthermore, TNF-α dramatically increased the movement as well as the directionality of ESCs. These results demonstrate that ESCs are highly motile and respond to different concentrations of calcium in a dose-related manner.

INTRODUCTION

There is increasing interest in stem cell transplantation as a potential therapy for improving the prognosis of patients with cardiac failure [1-3]. We have previously shown that after systemic injection, murine ESCs home to the hearts of mice with inflammation due to viral myocarditis [4]. However, the mechanism by which stem cell migration occurs remains largely unstudied.

Cell migration depends on morphologic changes that are controlled by proteins that are associated with the actin cytoskeleton. Calcium plays an important role in the regulation of actin-binding proteins. Calcium has also been shown to be involved in the stimulation of myosin II-based contraction [5] and in mediating the amount of integrin available to participate in cell migration [6]. Regulation of cyclic nucleotide levels is an essential element in modulating cell migration. Cyclic adenosine 3,5-monophosphate (cAMP) is a ubiquitous second messenger that regulates many cellular processes and has been shown to be required for efficient cell migration [7]. In addition, cyclic guanosine 3-5'-monophosphate (cGMP) has been discovered to modulate cell migration as well and is known to act through different proteins, affecting the extracellular matrix and actin cytoskeleton [8,9]. Tumor necrosis factor-α (TNF-α) is a cytokine that is released after cardiac injury, including myocardial infarction [10]. We recently showed that TNF-α enhanced migration of ESCs *in vitro* [11].

We hypothesized calcium and cyclic nucleotides affect the morphology and membrane deformation of ESCs and in addition TNF-α may play an important role in attracting ESCs. Here, therefore, we studied the effects of changes in extracellular [Ca^{2+}], cAMP and cGMP in the medium on intracellular [Ca^{2+}] and stem cell migration, using a simple mathematical model that allowed us to analyze the overall effect of these second messengers.

MATERIALS AND METHODS

Cell culture

Experiments were conducted using murine ESCs, ES-D3, obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, Logan, UT), 10% Fetal Bovine Serum (ATCC, Manassas, VA), 1% penicillin-streptomycin (Cellgro, Herndon, VA), 0.1% 2-mercaptoethanol (Invitrogen, Carlsbad, CA) and 1000 U/ml leukemia inhibitory factor (Sigma-Aldrich, Saint Louis, MO). The 6-wells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2. Growth medium was changed twice weekly and cells were subcultured weekly by dissociation with trypsin for 3 minutes.

Preparation of cells

Before each experiment cells were trypsinized for three minutes and suspended in growth medium. After an incubation period of 30 minutes the stem cell suspension was centrifuged at 1000g for 3 minutes. Collected cells were resuspended in DMEM, supplemented with 0.1 mM, 1 mM, 2 mM or 5 mM of Ca^{2+}, just before the start of the experiment. For the experiments with dbcAMP (1 mM) or dbcGMP (1 mM) the medium contained 0.1 mM of calcium.

Dye loading and calcium measurement

ESCs in suspension were loaded for 40 minutes with 5 μmol/l fura-2/AM (Molecular Probes, Eugene, OR). Extracellular fura-2/AM was washed away and the cells were resuspended in DMEM, with pH adjusted to 7.4. To determine the effect of dbcAMP (1 mM) and dbcGMP (1 mM) on he intracellular calcium concentration, these substances were added to the medium (~40 cells from 5 separate cultures per condition). Fluorescence intensity was converted to intracellular calcium concentration ([Ca^{2+}]i) using the following equation of Grynkiewicz et al. [12]:

$$[Ca^{2+}]_i = K_d\, b\, (R-R_{min})/(R_{max}-R)$$

where K_d is the dissociation constant of the Ca^{2+}/Fura-2 complex (225 nM), $b=F_{380}$ (zero Ca^{2+})/F_{380} (saturating Ca^{2+}), $R=F_{340}/F_{380}$, R_{min} represents the ratio at zero calcium (1 mM EGTA), R_{max} is the ratio at high calcium (1 mM $CaCl_2$, 5 μM ionomycin).

Setup of chemotaxis chamber

The Dunn chemotaxis chamber (Weber Scientific International Ltd., Teddington, UK) was filled with the cell suspension and covered by a 22x25 mm coverslip, leaving a narrow gap at one edge for refilling the outer well. The medium was then drained out of the outer well and refilled with medium containing 5 ng/ml of TNF-α. Finally, the gap was sealed using a mixture of hot wax (vaseline, beeswax, paraffin wax 1:1:1).

Recording and tracking cell behavior

A region of the bridge of the Dunn chamber was viewed under an inverted fluorescence microscope at a magnification of 4x (TE-2000-U, Nikon, Tokyo, Japan). Images were obtained every two minutes for a three-hour period with a Photometrics Cool Snap HQ charge-coupled device camera (Roper Scientific, Trenton, NJ).

Fura-2 fluorescence was excited alternately at 340 and 380 nm using a filter wheel and a 75 W Xenon lamp. To reduce photobleaching, a shutter prevented illumination of the cells, except during data acquisition. The fluorescent emissions were acquired through a 510-nm dichroic mirror and 520 nm long pass filter set (Chroma Technology, Brattleboro, VT). Acquired images were generated at 6-second intervals and background was subtracted from each image. The loaded cells were viewed using a 20x objective (Nikon, Tokyo, Japan). Images were analyzed by IP Lab software (Scanalytics Inc., Fairfax, VA). Using the centroid of the cell, each position of the cell was obtained throughout every image to measure cell distance. Speed was calculated by dividing the cell distance by the duration of the movement. The directionality of the migrating cells was determined by using scatter diagrams. In the scatter diagram, the outer well, which contained the TNF-α, is represented by the y direction. The starting point of the cells is the intersection of the y- and x-axes (0,0), the data points correspond with the final position of the individual cells.

Morphological analysis

The Dunn chamber was set up as described previously (20 cells from 5 different cultures per condition) Using an inverted fluorescence microscope at a magnification of 40x, we were able to study the morphology of ESCs Images were obtained every 6 seconds for a period of 60 minutes

Statistical analysis

Results are presented as mean±S E M Comparison between groups was done using one-way ANOVA When significant group differences were observed, Student's two-tailed *t*-test for unpaired observations was performed Differences were considered significant with $P<0\ 05$

RESULTS

Mathematical model

A measure of cell mobility should depend amongst other factors on the speed a cell is traveling at, the duration it is traveling for, as well as the distance traveled in the direction of the chemical gradient One way to express mobility for a cell population is to compute averages of the aforementioned quantities over the cell population While this has the benefit of maximal information content it is difficult to interpret A single scalar quantity as a measure of cell mobility is thus favorable One way of combining the multiple measured cell state quantities into such a single scalar quantity is by weighting of the individual influence terms However, this results in various weighting constants that need to be determined Borrowing from variational mechanics this paper thus uses action as a measure for cell mobility The action, A, of a system between time point t_1 and t_2 is defined as the time integral over the Lagrangian function $L(x(t),d/dt\ x(t), t)$, where x_t denotes position, $d/dt\ x_t$ velocity, and t time, i e ,

$$A = \int_{t_1}^{t_2} L(x(t), \dot{x}(t), t)dt.$$

The action is a measure of the time integral of the dissipated energy (up to a constant) Choosing the Lagrangian as the difference between kinetic (T) and potential energy (U), the action may be written as

$$A = \int_{t_1}^{t_2} T - U dt.$$

Regarding the cell as a point mass, m, with the potential gradient given by the chemical gradient, c, towards the wall the action integral becomes

$$A = \int_{t_0}^{t_1} \frac{1}{2} m\dot{x}^2 - (-cx)dt.$$

To compute the action integral exactly, the cell speed and position need to be measured at every time instant, t. To facilitate computations we assume the cell is traveling for a time t_m at the constant speed v. and that the chemical gradient is constant. With $t_1=0$ and $t_2=T$ (overall measurement time) some algebraic manipulations of the action integral result in

$$A = \frac{1}{2} v t_m (v + \frac{c}{m} t_m) + \frac{c}{m} d(T - t_m)$$

which is the sought for expression for cell mobility. The first term becomes large for cells traveling at high average speeds over long periods of time. The second favors large displacements in the direction of the chemical gradient over short periods of time. Thus, large values of A will indicate high cell mobility.

Calcium increases migration

To investigate whether calcium has an effect on stem cell migration, we performed the migration assays with different concentrations of extracellular Ca^{2+} ($[Ca^{2+}]_e$), 0.1 mM, 1 mM, 2 mM and 5 mM (~60 cells from seven separate cultures per condition). We found a significant concentration-dependent increase in the speed of migration (Fig. 1A).

In addition, cells moved significantly further and longer when a higher concentration of calcium was added to the medium ($P<0.05$; Fig. 1B). Experiments with the fluorescent Ca^{2+} indicator, fura-2/AM, demonstrated that increased $[Ca^{2+}]_e$ significantly increased $[Ca^{2+}]_i$ (40 cells from five separate cultures per condition, Fig. 2A). However, the most remarkable increases in $[Ca^{2+}]_i$ were found in cells that were about to migrate or were migrating (Fig. 2B). These results indicate that calcium plays an important role in the migration of embryonic stem cells.

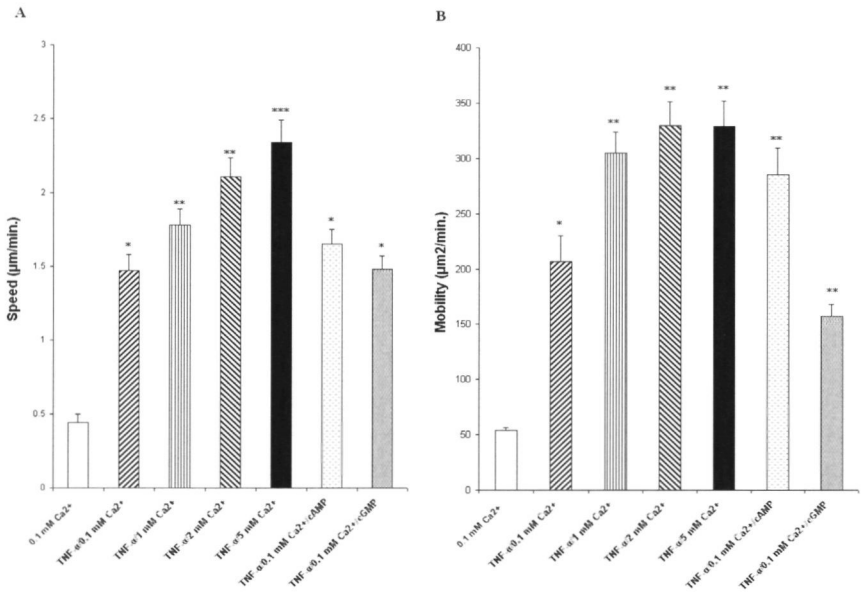

Figure 1. Calcium and cAMP but not cGMP stimulate TNF-α induced migration of ESCs. **(A)** TNF-α and calcium increased ESCs speed of migration compared to controls. Administration of cAMP or cGMP did not affect cell speed. Speed of migration was determined for each condition by dividing total distance by the total time of migration. **(B)** Calcium and cAMP stimulated and cGMP diminished ESC mobility. ESCs subject to TNF-α were significantly more mobile than the control cell population. (Mean±S.E.M., **P*<0.05 vs. control, ***P*<0.05 vs. TNF-α plus 0.1 mM Ca^{2+}, ****P*<0.05 vs. TNF-α plus 1 mM Ca^{2+}, ~60 cells from 7 separate cultures per condition.)

Cyclic AMP stimulates embryonic stem cell migration

It has previously been shown that cAMP can have different effects on migration of many different cell types. We found that dbcAMP had a positive effect on stem cell migration. With regard to mobility, we found an average of 207±23 µm with 1 mM dbcAMP, compared to a 286±24 µm with only 0.1 mM Ca^{2+} (*P*<0.05, ~60 cells from 7 separate cultures per condition). Interestingly, this computed mobility, was comparable to those with high extracellular calcium, however speed was not. The speed of migration (Fig. 1A) did not significantly increase in the experiments with 1 mM dbcAMP added (*P*>0.05). Therefore, speed appears to be dependent on a threshold amount of calcium being present in the medium and proves to be the most sensitive parameter in distinguishing the effect of different calcium concentrations on migration.

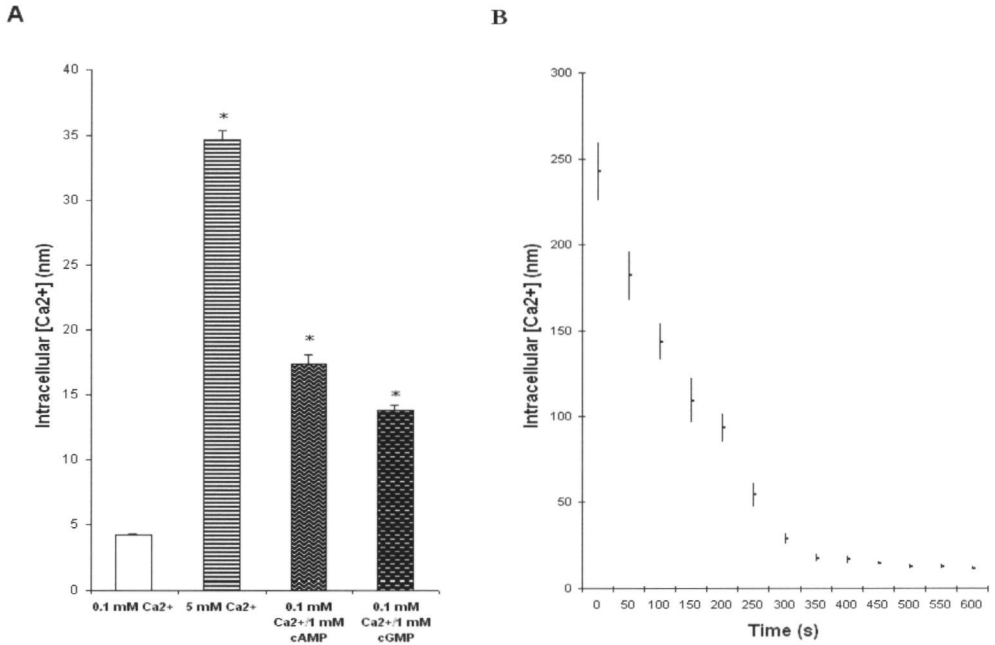

Figure 2. Changes in intracellular calcium $[Ca^{2+}]_i$ concentration. A. Increase in intracellular $[Ca^{2+}]$, induced by extracellular calcium, dbcAMP and dbcGMP. At least 40 cells from five separate cultures were analyzed per condition, ratio was calculated every 6 seconds.
*P<0.05 vs. control B. Changes in $[Ca^{2+}]$i of migrating stem cells. The increase in $[Ca^{2+}]$i compared to quiescent cells demonstrates to be of temporary nature as it is pumped out of the cell during the time of migration. (Mean±S.E.M., ~20 cells from five separate cultures).

We examined whether the effect of cAMP on the migration of stem cells is related to its effect on the intracellular calcium concentration. We found that cAMP significantly increased the $[Ca^{2+}]_i$ from 4.2±0.06 nM to 17.4±0.7 nM in ESCs (Fig. 2A). Also, we found a significant difference in the $[Ca^{2+}]_i$ between 5 mM Ca^{2+} and 1 mM dbcAMP (Fig. 2A). We believe this difference might explain the difference in migratory speed induced by these two agonists.

Cyclic GMP affects the mobility of stem cells

We assessed whether cGMP had an effect on migration of ESC. For this we added 1 mM dibutyryl cGMP to the medium. We found no effect with dbcGMP on cell speed compared to

dbcAMP or 0.1 mM calcium ($P>0.05$, Fig. 1A). Mobility, however, was significantly reduced after adding dbcGMP ($P<0.05$, ~60 cells from seven separate cultures per condition). In order to determine whether this depressant effect of cGMP on the mobility of stem cells was related to the calcium concentration of the cell, we measured the ratio of fura-2 after adding dbcGMP to the medium. We found that dbcGMP did increase $[Ca^{2+}]_i$, but not to the same extent as dbcAMP (Fig. 2A).

TNF-α induction of proteopodia

The addition of 5 ng/ml TNF-α to the outer well of the Dunn chamber, resulted in a dramatic increase in cell movement (Fig. 1). To further demonstrate the role of TNF-α in directing stem cell migration, we plotted cell migration in a scatter diagram (Fig. 3).

The data points indicate the final positions of the cells after three hours of recording and show that TNF-α gradients stimulate ESC chemotaxis (Fig. 3). In TNF-α-free setups, the migration was not directionally orientated.

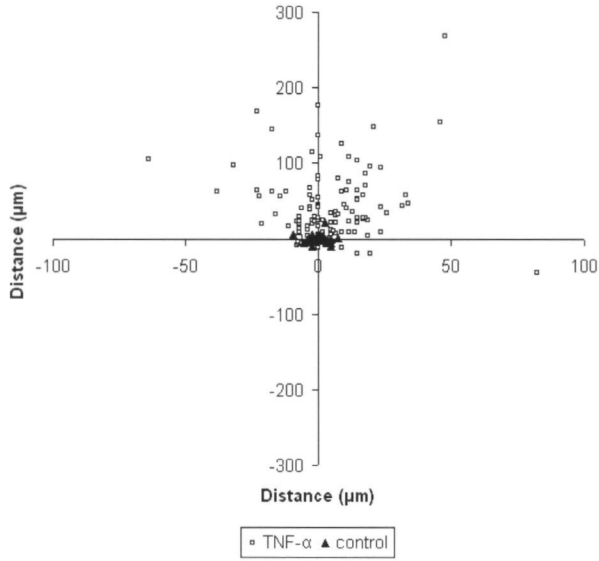

Figure 3. Effect of TNF-α on the directional migration of ESCs. The starting point for each cell is at the intersection between *x* and *y* axes (0,0) and data points show the final location of individual cells after the 3-hour recording period. The source of TNF-α (5 ng/ml) is at the top. Data of five separate cultures are shown, at least 50 cells per condition.

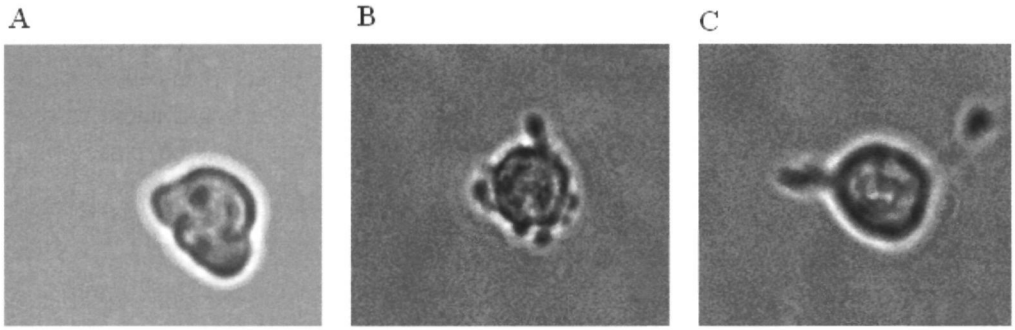

Figure 4. Representative images of migrating embryonic stem cells with different membrane extensions. The experiments were performed in the presence of TNF-α as chemotactic agent. **(A)** Embryonic stem cell expressing lamellipodia (40x). **(B)** ESC with short proteopodia (40x). **(C)** Embryonic stem cell expressing long proteopodia (40x). **(D)** Movie of ESC that displays chemotaxis towards a TNF-α gradient (4x).

In experiments where we studied the morphology of ESCs, we found that under the influence of TNF-α, ESCs expressed different types of membrane extensions (~20 cells from five different cultures per condition). We detected lamellipodia (Fig. 4A) and filopodia as well as the less described proteopodia [13], which were either short and club-like or long and slender (Fig. 4B and C).

The long proteopodia were able to change rapidly, extending and retracting, in search for contact. However, for migration, the short proteopodia appeared to be an important factor, since only motile cells expressed those.

DISCUSSION

The central role of calcium in mediating the movement of various cell types, including adult and fetal stem cells, is well established [14-17]. This study, to our knowledge, is the first to show that the second messenger pathways mediating movement of more differentiated stem cells are also present and functional in embryonic stem cells. Our results demonstrate that a pathway involving calcium regulates the migration of ESCs. Distinct increases in intracellular calcium levels were observed, immediately before and during migration of ESCs. Increases in extracellular calcium levels also enhanced migration.

Calcium is known for its effect on movement of cells. The normal intracellular calcium concentration is about 0.1 μM but can rise to 10 μM after release from internal stores. The change in $[Ca^{2+}]_i$ can result in movement and associated changes in the cytoskeleton. Calcium initiates cytoskeletal changes by binding and changing the activity of a cytoskeletal protein, such as calmodulin or troponin, which possess calcium-binding domains [18,19].

To date, most studies about cardiac repair with stem cells have employed adult stem cells [20-22]. However, experimental and theoretical considerations suggest that ESCs may have advantageous properties for tissue regeneration. Better knowledge of the ways of modulating the homing, transmigration and implantation of ESCs is needed for the attainment of therapeutic goals.

Cyclic nucleotides have been shown to modulate the migration and homing of various cell types and we therefore treated and studied the ESCs with dbcAMP and dbcGMP. We found that dbcAMP increased mobility of the cells. As expected, we found that dbcAMP raised the intracellular calcium concentration. Cyclic AMP is a second messenger that activates protein kinase A (PKA) and is known to be involved in many cell functions, as well as cell shape and organization of actin cytoskeleton. PKA on activation in turn organizes actin in bundles [6,7,23].

As in the case of cAMP, cGMP has been described in the literature as having inhibitory as well as stimulatory effects on cell migration. Elferink et al. explain this discrepancy by claiming that the concentrations of cGMP used in experiments and conditions such as the existence of extracellular calcium and chemotactic agents, determine whether cGMP will have a stimulatory or inhibitory effect on migration [24]. Our data suggest that cyclic GMP has a depressant effect on stem cell migration. Cyclic GMP is an important second messenger, with several intracellular targets. It can bind to protein kinase G (PKG), which is known to suppress the production of the extracellular matrix proteins, which in turn are needed in the migration and proliferation of several cell types such as smooth muscle cells and HUVEC cells [8,9,25]. Furthermore, cyclic GMP is known for mediating most actions of nitric oxide (NO) that in turn is implicated in inhibiting the formation of F-actin [26,27]. These results are important because they indicate that factors altering the intracellular levels of $[Ca^{2+}]_i$, cAMP and cGMP may modulate the homing, transmigration and attachment of ESCs to desired sites of repair and regeneration in organs, including the heart.

We were able to study the migratory patterns at cellular level by using videomicroscopy. To increase cell speed, morphological changes and minimal adhesion are necessary [28,29]. We achieved the maximum speed by not coating the cells to a coverslip and by using calcium to change the morphology. Since we found that the presence of a threshold amount of $[Ca^{2+}]_e$ is necessary for reaching high speeds, calcium can be valued as an important factor for the homing of ESCs. As for directionality, the influence of TNF-α on stem cell migration was

proven by their chemotactic response to concentration gradients of TNF-α (Fig. 3), hence confirming the previous findings of our group that TNF-α attracts ESCs [11]. Using videomicroscopy, we were able to study the morphology of the moving cells. We distinguished several forms of extensions, such as filopodia, lamellipodia and proteopodia. Previously, proteopodia have been described in a study of haematopoietic stem cells (HSC), where they were associated with directed motility, homing and engraftment of HSCs [13]. In our setup, these proteopodia were only observed when TNF-α was used in the experiments, implicating an important role of TNF-α in the expression of these proteopodia and in inducing stem cell migration.

In summary, we have shown that calcium and cyclic nucleotides play an important role in mediating the movement of embryonic stem cells. We plan to further investigate the actions and interactions of calcium, cAMP and cGMP to understand these cellular mechanisms in anticipation of being able to use these to enhance the use of stem cell transplantation.

ACKNOWLEDGEMENTS

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CHAPTER 3

Successful implantation of intravenously administered stem cells correlates with severity of inflammation in murine myocarditis

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ABSTRACT

The present study was designed to determine whether cardiac inflammation is important for successful homing of stem cells to the heart after intravenous injection in a murine myocarditis model. Male BALB/c mice were infected with encephalomyocarditis virus (EMCV) to produce myocarditis. Subgroups of mice received single injections by tail vein of embryonic stem cells (ESCs) transfected with green fluorescent protein (GFP) as a marker at days 3, 14 or 60 after infection; other subgroups without stem cell injection were sacrificed at each of these time points to assess the degree of inflammation present. Surviving mice were sacrificed at day 90 after virus infection and hemodynamics, gross pathology, histology and inflammatory cytokine production in the hearts were measured. Our results indicate that myocardial inflammation was most severe and cytokine production highest at day 14 after EMCV inoculation, and in particular was strongly positive for interleukin 6. Mice receiving intravenous ESC injections on day 14 after EMCV inoculation showed the largest number of GFP positive cells at the time of sacrifice and the greatest functional improvement compared to uninfected controls without inflammation. We conclude that factors released from myocardium during inflammation are important for enhancing the homing, migration and implantation of systemically infused stem cells.

INTRODUCTION

Direct cellular transplantation of various types of stem cells in the heart has shown a remarkable ability to produce sustained improvement in cardiac structure and function [1-5]. The majority of these reports are in the setting of the acute myocardial infarction (MI), raising the possibility that the factors up-regulated during MI are critical to the success of stem cell transplantation and subsequent proliferation and differentiation into functional myocytes.

We reported that stem cell transplantation can significantly improve the survival rate and reduce necrosis in a model of viral myocarditis [6]. Thus, it is possible that inflammatory factors up-regulated during myocarditis are critical to the success of cell transplantation. Our previous study indicated that viral myocarditis is characterized by cardiac necrosis and inflammation in the acute stage, followed by necrosis, inflammation, myocardial fibrosis, calcification, and cardiac dilation in the chronic stage [6]. Our *in vitro* data demonstrated that excessive tumor necrosis factor–alpha (TNF-α), an inflammatory cytokine that is up-regulated in the setting of cardiac inflammation and dysfunction, enhances migration of embryonic stem cells (ESCs) [7]. The present study was designed to test the hypothesis that cardiac

inflammation is important for successful homing of stem cells to injured heart after intravenous injection in a murine model of encephalomyocarditis virus (EMCV) induced myocarditis established in our laboratory Mice were inoculated with EMCV and then injected with mouse ESCs by tail vein 3, 14 or 60 days later Morbidity, mortality, histopathology and cardiac function were assessed to evaluate the success of ESC homing Our results support the hypothesis that the presence of inflammation can profoundly affect the structural and functional response of the heart to engrafted stem cells delivered via intravenous infusion, and this effect may be modulated by locally produced inflammatory cytokines

MATERIALS AND METHODS

Virus preparation and inoculation of mice

BALB/c male mice aged 6 weeks were obtained from Charles River Laboratory Experiments with these animals conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No 85-23, revised 1996) The Institutional Animal Care Committee of Beth Israel Deaconess Medical Center approved our experimental protocol Seventy-two of these mice were inoculated with the M variant of EMCV (ATCC, Manassas, VA) Briefly, human amnion (FL) cell monolayers were infected with virus and harvested when cytopathic effects were completed The viral titers were determined by plaque formation on FL cell monolayer The viral stock was stored at -80°C until use Mice were inoculated intraperitoneally (ip) with 140 plaque-forming units (0 1 ml) of virus diluted in Eagle's minimum essential medium

Stem cell preparation and intravenous infusion

The mouse ESC line, ES-D3, was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained with methods used in previous experiments [3-5] Briefly, ES-D3 cells were cultured in DMEM on mitotically inactive mouse embryonic fibroblast feeder cells The medium was supplemented with 15% fetal bovine serum, 0 1 mM β–mercaptoethanol and 10^3 units/ml of leukemia inhibitory factor conditioned medium (BRL, Gaithersburg, MD) to suppress differentiation To initiate differentiation, ESCs were dispersed with trypsin and resuspened in the medium without supplemental leukemia inhibitory factor and cultured with a hanging drop method (approximate 400 cells/20 μl) for 5 days They were then seeded into 100-mm cell culture dishes Spontaneously beating clusters were dissected with a sterile micropipette and recultured for another 2-3 days Before cell infusion, ESCs were transfected with enhanced green fluorescent protein (GFP) for

identification of infused ESCs. A plasmid with hCMV IE promoter/enhancer driving GFP gene (5.7 kb) and GenePORTER^{TM} transfection reagent was obtained from Gene Therapy Systems Inc. (GTS Inc., San Diego, CA). The transfection efficiency is over 90%. Two days after GFP transfection, cultured ESCs were trypsinized and resuspended in Joklik modified medium (Sigma) with a density of 10^7 cells/ml for infusion.

Three days after virus inoculation, 12 randomly selected mice were separated and 150 µl medium suspensions (containing $1.5x10^6$ cells) were injected into the tail vein of each mouse. On the same day, 8 additional randomly selected mice were sacrificed. Heart, spleen, liver, lung, and kidney were excised and stored in 10% formalin solution. These tissues were then stained with hematoxylin and eosin for pathology scoring. Blood was also taken from the vena cava of each sacrificed mouse. This same procedure was repeated on days 14 and 60 after EMCV inoculation.

Pathology scoring

For mice sacrificed at 3, 14 and 60 days after virus inoculation, three transverse sections of the left ventricle were fixed in 10% formalin and embedded in paraffin, sectioned at 5 µm thickness and stained with hematoxylin and eosin. An experienced pathologist scored 3 sections of each heart blindly. For each myocardial sample (mice whose deaths were unobserved did not undergo necropsy), histological evidence of myocarditis and inflammation was classified in terms of the degree of cellular infiltration and myocardial cell necrosis and graded on a 5-point scale ranging from 0-4+ [6]. A zero score indicated no or questionable presence of lesions in each category. A 1+ score described a limited focal distribution of myocardial lesions. A score of 2+ to 3+ described intermediate severity with multiple lesions, whereas a 4+ score described the presence of coalescent and extensive lesions over the entire examined heart tissue.

Hemodynamic measurements and GFP detection

In another set of experiments, on day 90 after virus inoculation, 5 mice from each group were anesthetized with ketamine (50 mg/kg ip) and xylazine (2.5 mg/kg ip), and hemodynamic measurements were performed to evaluate heart function. A Millar catheter (1.4-F Millar, Millar Instrument, Houston, TX) was inserted into the carotid artery and advanced into the left ventricle. Heart rate, left ventricular end diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), and maximum rate of left ventricular systolic pressure rise (dP/dt_{max}) were recorded using Power Lab data acquisition equipment and software (AD Instruments, Colorado Springs, CO). After hemodynamic measurements mice were sacrificed and the hearts were excised and stained with hematoxylin and eosin for necrosis scoring [6]. Additionally, different frozen sections from another batch of

experimental mice (5 for each) were stained immunohistochemically with a mouse monoclonal anti GFP antibody (Zymed, San Francisco, CA) Fluorescent staining of GFP was detected and photographed under confocal microscopy

Cytokine staining and cytokine gene array

Cytokine staining was performed for the hearts excised from mice sacrificed at 3, 14 and 60 days after virus inoculation Xylene was used to remove paraffin fixed heart sections and following dehydrated with 100%, 95%, 80% and 40% of ethanol, 5 min for each procedure To block endogenous peroxidase, the slides were then incubated in 1% H_2O_2 for 10 min at room temperature Antibody against signal pathway phospho-ErK, JNK and p38 (1 100, Cell Signal Technology, MA) or antibodies against cytokines IL-6, IL-10, TNF-α and TNF-β (1 50, Chemicon, CA) were applied and the sample was incubated at 4°C overnight This was followed by peroxidase-labeled antibody and diaminobenzidine as a substrate, which imparted a yellow-brown color to the positive cells A micro-array analysis (3 for each group) was performed on the hearts of representative mice that were sacrificed 14 days after inoculation in order to determine the cytokines expressed in viral myocarditis Total RNA was extracted from the heart tissue by Tri-Reagent (Sigma, MO) Two μg of RNA were used to perform cytokine analyses via the GEArray Q Series Mouse Common Cytokine Gene Array (SuperArray Bioscience Corporation, MD) The gene array assay (virus infected samples versus uninfected samples) was performed according to the protocol provided by the SuperArray Bioscience Corporation, and the data were analyzed with a GEArray analyzer Gene comparisons were expressed as a ratio adjusted for background and housekeeper gene expression A greater than 2-fold increase in the gene signal intensity was considered significant

Serum cytokine levels

Blood samples taken from mice at 3, 14 and 60 days after inoculation were centrifuged to obtain serum This serum was then analyzed using the quantitative sandwich enzyme immunoassay technique for IL-10, IL-6, TNF-α and TNF-β (R&D systems Quantikine, Minneapolis, MN) A monoclonal antibody specific for each mouse cytokine of interest was pre-coated onto a microplate Standards and controls, as well as the serum samples, were pipetted into the wells and the immobilized antibody bound the specific mouse cytokine After washing, enzyme-linked polyclonal antibody against the cytokines was added to the wells The enzyme reaction yielded a blue product that turned yellow when the stop solution was added The intensity of the color was measured in proportion to the amount of mouse cytokine bound in the initial step The sample values were then read off a standard curve

Statistical analysis

Results are presented as mean±standard error or in bar graph form with positive standard error bars. Data derived from three or more animals were evaluated by ANOVA with repeated measurements. Comparison within and between groups was performed by using paired or unpaired Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Inflammation score

We analyzed the myocardium of the mice sacrificed at 3, 14 and 60 days after virus inoculation, and discovered that myocardial inflammation was greatest at 14 days after virus inoculation (Fig. 1A-E).

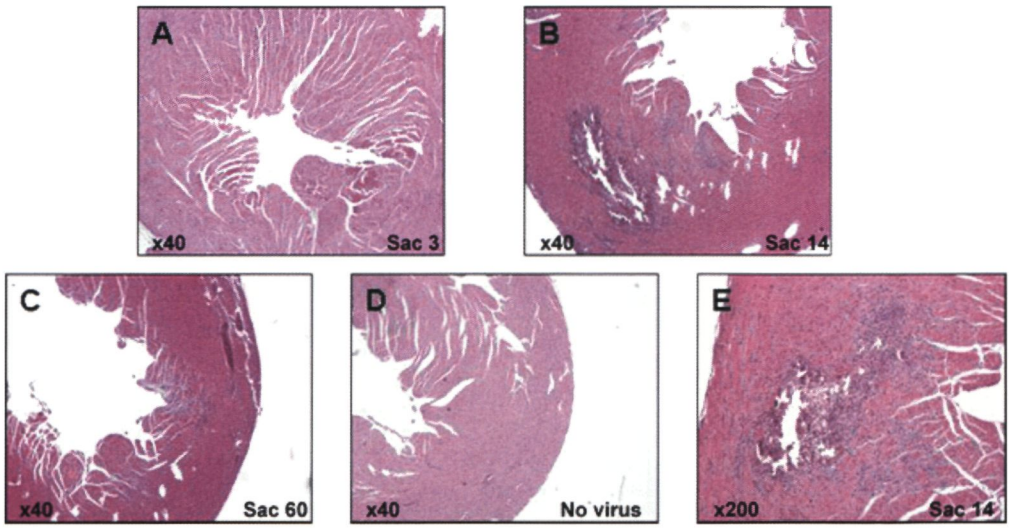

Figure 1. Hematoxylin and Eosin stained sections of mouse hearts excised from the animals sacrificed 3 (**A**), 14 (**B**), and 60 days (**C**) after EMCV inoculation as well as control sample that received no virus (**D**). Inflammation was greatest at day 14 (**B**). Panel **E** shows high power view of the lesion caused by the virus 14 days after virus inoculation. This type of lesion shows lymphocyte and mononuclear cell infiltration surrounding and between necrotic cardiomyocytes. The mononuclear cells are large with vesicular nuclear chromatin patterns and appear to be activated.

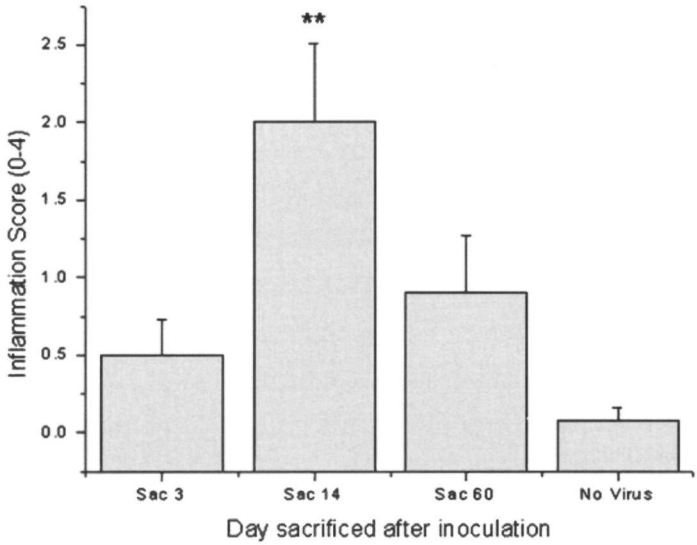

Figure 2. Inflammation score at different days for the mice inoculated with EMCV (8 for each group). Histological scoring of hearts ranged from 0 to 4+ in each of the categories of inflammation and necrosis. $^{**}P<0.01$ vs. other three groups.

The myocardial inflammation demonstrated on this day was significantly greater than the other groups treated with stem cells on days 3 or 60 after inoculation (Fig. 2).

Cytokine detection

Serum cytokine levels were significantly elevated in the virus inoculated animals. Although we observed evidence of increased levels of many cytokines in virus infected animals, including IL-10, TNF-α and TNF-β, the factor that showed the most correlation with inflammation at the time of stem cell infusion was IL-6. When stained with IL-6 antibody, the mice sacrificed 14 days after inoculation showed the most frequent and extensive patches of IL-6 positive stains on the heart sections (Fig. 3A).

The mice sacrificed 14 days after virus inoculation displayed significantly higher serum levels of IL-6, IL-10, TNF-α and TNF-β than any other group (Fig. 3B to 3E, respectively). Micro-array analysis showed that the RNA isolated from heart tissue excised from an EMC virus infected mouse 14 days after inoculation had an increased expression of IL-6, IL-10 and other cytokines compared to an uninfected mouse (Table 1).

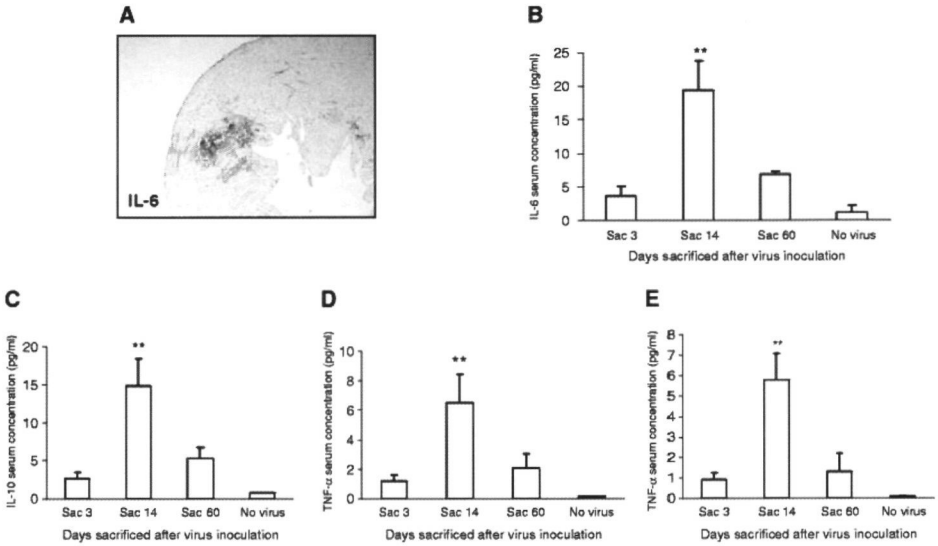

Figure 3. IL-6 staining of heart sections excised from mice sacrificed 14 days after virus inoculation (**A**). The stains appear yellow-brown and were most intense in the areas of greatest inflammation. The bar graphs represent the average serum concentrations of IL-6 (**B**), IL-10 (**C**), TNF-α (**D**) and TNF-β (**E**), respectively, for mice sacrificed 3, 14 and 60 days after inoculation, along with the mice that received no virus (8 for each group). $^{**}P<0.01$ vs. other three groups.

Table 1. Gene exhibiting a twofold or greater increase in heart tissues from virus infected mice versus uninfected mice

Gene description	Symbol	Expression ratio (Virus infected samples / uninfected samples)
Interleukin 6	IL-6	4.7
Interleukin 10	IL-10	3.4
Interleukin 18	IL-18	3.4
Interleukin 12B	IL-12B	3.1
Colony stimulating factor 2 (Granulocyte-macrophage)	GM-CSF	3.1
Interleukin 7	IL-7	2.9
Interleukin 13	IL-13	2.8
Interleukin 3	IL-3	2.7
Interferon gamma	IFN-r	2.7
Interferon alpha family, gene 4	IFNA5	2.5
Tumor necrosis factor-α	TNF-α	2.3
Lymphotoxin B	TNF-β	2.1
Interleukin 1β	IL-1β	2.0
Interleukin 5	IL-5	2.0
Interleukin 11	IL-11	2.0

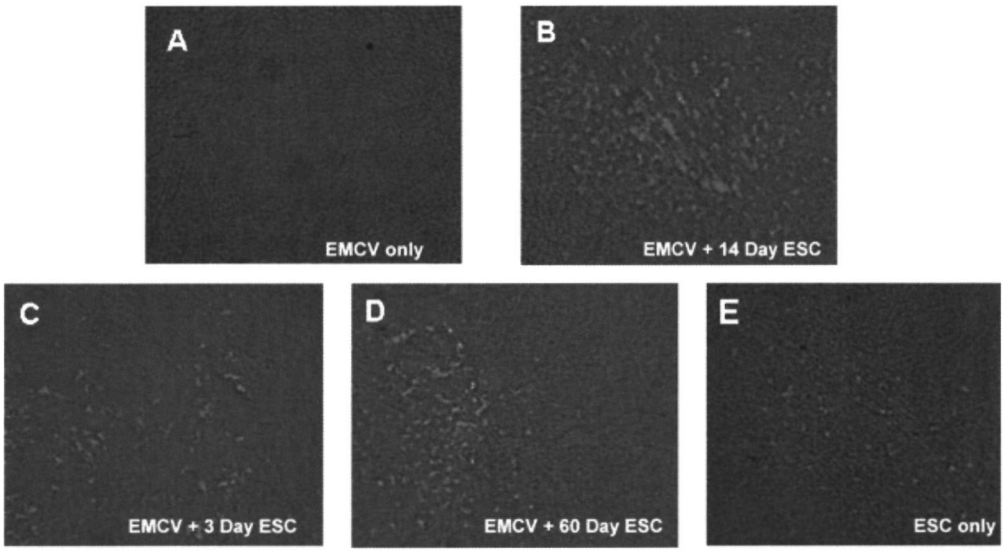

Figure 4. 100x magnification images of left ventricular myocardium sections excised from each of the four intravenous stem cell treatment groups as well as the group inoculated with EMCV but not treated with intravenous ESC infusion (**A**).
The ESCs express GFP, and appear green when photographed under fluorescent light. Panel **B** is from a mouse heart treated with stem cells at 14 days after EMCV inoculation, and displays a wide field of GFP expressing cells. Panel **C** was from a mouse heart treated with ESCs 3 day after virus inoculation, while panel **D** was from a mouse heart treated with ESCs 60 day after virus inoculation, and both display a smaller field than the 14-day treated group. The final image (**E**) was from the ESC treated mouse hearts at 14 days after cell infusion that was not inoculated with EMCV as ESC-treated control. There is a trend of the 14-day stem cell treated group had more GFP positive cells presented in virus-infected myocardium than all other groups.

GFP detection to identify donor ESCs

GFP cells were detected in all the hearts of ESC treated groups (Fig. 4). Traces of scarcely GFP were also detected in the liver, spleen, and kidney of the mice that received intravenously infused stem cells, only spleen showed a few GFP positive spots (data not shown). Compared to mice without EMCV inoculation, the EMCV myocarditis mice had a significantly greater number of GFP positive cells in the myocardial tissue after intravenous infusion of ESCs. Furthermore, mice treated at day 14 after virus infection displayed the most GFP positive cellular spots in their myocardial tissue (Fig. 4B), indicating the maximum amount of ESCs homing to injured myocardium in this group.

Table 2. Hemodynamic measurements for stem cell treated and control mice were obtained 90 days after EMCV inoculation

	LVSP (mmHg)	LVEDP (mmHg)	Heart Rate (beats/min)	dP/dt_{max} (mmHg/s)
Virus only	81±6	5 85±2	345±31	3046±330
Virus + ESC 3 day	92±9	3 94±1	358±26	3292±857
Virus + ESC 14 day	99±2*	2 96±1*	406±39*	3974±953
Virus + ESC 60 day	94±3*	3 72±2	395±72	3571±177*
ESC(no Virus)	100±4*	1 35±2*	430±60*	4089±632*

$N \geq 4$ for each group *$P<0$ 05 versus virus only group LVSP, left ventricular systolic pressure, LVEDP, left ventricular end diastolic pressure, dP/dt_{max}, maximum rate of left ventricular systolic pressure rise

Assessments of cardiac function and necrosis score

Cardiac function was assessed by hemodynamic measurements in experimental mice and the data were shown in Table 2 All stem cell treated groups demonstrated a trend towards better hemodynamic performance than the virus-only group LVSP and dP/dt_{max} were significantly improved in the groups that received intravenously infused stem cells at 14 and 60 days after virus inoculation as compared to the virus only group that did not receive stem cell infusions However, the improvement in LVSP for 14-day stem cell infusion was significantly greater ($P< 0$ 05) than that of the 60-day group (Table 2)

At 90 days, all groups that received intravenously infused stem cells at different time points showed significantly lower necrosis scores (Fig 5, following page) than the EMCV group without stem cell treatment The group that received cell infusion 14 days after virus inoculation showed the least necrosis (Fig 5)

DISCUSSION

Our study demonstrated that the presence of cardiac inflammation appears to be critical for successful ESC transplantation when cells are administered systemically into the blood stream Homing, implantation, transmigration, proliferation and functional improvement due to ESC infusion, correlated with the maximal amount of inflammation of EMCV myocarditis This suggests that cardiac inflammation and its associated factors are critical for successful implantation of intravenously infused ESCs

Successful transplantation of stem cells depends upon the distribution, migration and homing of cells to the appropriate organ and their localization in the appropriate microenvironment that facilitates survival and functional improvement Homing, defined as

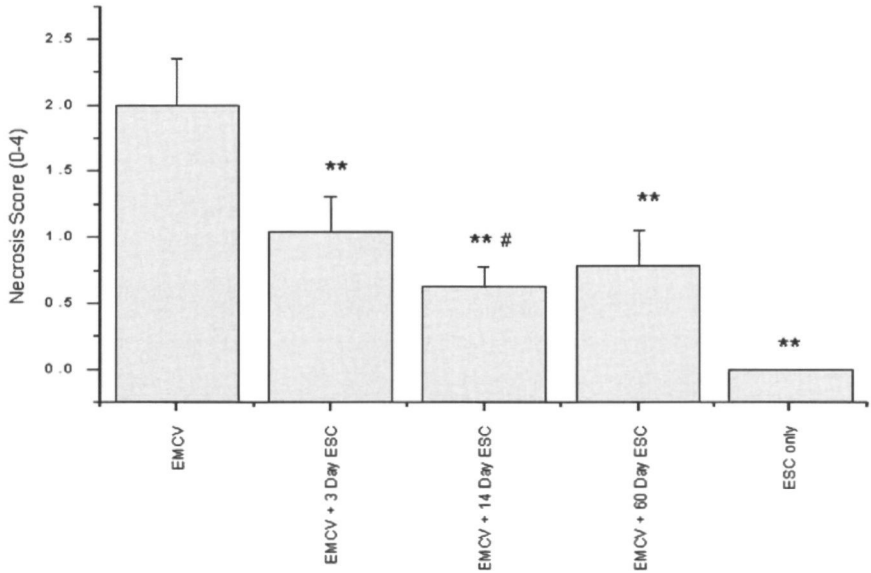

Figure 5. The bar graphs represent the average necrosis score (5 for each group) from mice sacrificed 3, 14 and 60 days after inoculation, along with the mice that received no virus. The mouse myocardium 14 days after virus inoculation and receiving stem cell infusion showed the least necrosis. $^{**}P<0.01$ vs. EMCV; $^{\#}P<0.05$ EMCV +14 Day ESC vs. EMCV +3 Day ESC or ESC only.

the recognition and binding of infused cells in a specific stromal microenvironment [8], has been most thoroughly studied for hematopoietic cells, or bone marrow derived stem cells (BMDCs). Studies have shown homing to the heart and the potential of BMDCs to regenerate myocardial tissue following MI [9]. A concentration gradient of stromal-cell-derived factor-1alpha (SDF-1α) is the major mechanism for homing of BDMCs. A recent study showed that following MI, treatment with SDF-1α plasmid recruited endogenous BDMCs to damaged heart where they may have a role in repair and regeneration [10]. Another group additionally looked at the effect of MI induced SDF-1 and showed that SDF-1/CXCR4 interactions play a crucial role in the recruitment of BMDCs to the heart after MI and can further increase homing in the presence, but not in the absence, of injury [11].

Inflammation plays a key role in the pathogenesis of cardiac disease [12]. Inflammatory cytokines like TNF-α and IL-6 have been shown to predict cardiovascular events [13]. It is likely myocardial dysfunction may also result in the appearance of cardiac receptor molecules that are important for attachment and long-term stability of stem cells in the

microenvironment [14-19]. Recently, it has been shown that enhanced expression of adhesion molecules (ICAM-1), chemoattractant cytokines (MCP-1), and matrix metalloproteinase (MMP) activity, on days 3 and 7 of post-MI, are present to facilitate the homing, chemotaxis, and migration of circulating cells into the infarct site [20]. It is interesting to note that a variety of compounds have been reported to attenuate the hematopoietic cell homing response, including IL-6, IL-1, thrombopoietin and granulocyte-colony stimulating factor [21-23] under some circumstances. However, our previous *in vitro* migration assay indicated that the cytokine, TNF-α, enhances migration of ESCs, an effect, that is mediated via stimulation of TNF-RII and activation of p38 and c-Jun amino-terminal kinase [7]. The present study shows that upregulation of cytokine, IL-6 appeared to be a crucial factor in this process.

We hypothesized that cardiac inflammation may play a role in attracting stem cells administered intravenously. We previously have shown improvement in heart function in a murine myocarditis model where stem cells were injected intravenously [6], and thereafter wanted to demonstrate the role of inflammation in our model. Our study clearly showed the propensity for homing of stem cells to the damaged heart. A consequence of decreased relative necrosis and improved heart function was most dramatic at the time of maximal inflammation. Furthermore, we showed that upregulation of cytokine, IL-6 appeared to be a crucial factor in this process.

Chemotaxis is the process of locomotion along a chemical gradient, probably initiated by the binding of chemotactic agents to specific receptors on the cell membranes of leukocytes, which activate second messenger systems. These chemoattractants include cytokines such as TNF-α and IL-6 [24,25]. Other factors shown to have chemotactic effects of leukocytes include platelet activating factor (PAF) [13] and nitric oxide (NO) [27,28]. Although the precise cellular steps of leukocyte versus stem cell movement may differ, it is reasonable to assume that some of the activators of the former, including IL-6, may also affect circulating stem cells in a similar fashion; e.g., it has been shown that IL-6 and IL-8 stimulate the migration of polymorphonuclear leukocytes, Langerhans cells [24,25] and vascular smooth muscle cells [29,30].

Our results show that the greatest success of cell transplantation occurred after intravenous infusion of ESCs at the time of the most severe degree of myocardial inflammation. We conclude that the factors released from myocardium during inflammation, in particular cytokines, are critical for the success of cell transplantation of ESCs delivered systemically. These results may be important in considering the likelihood of success of stem cell transplantation, particularly in patients without significant cardiac inflammation. Cells administered by the intravenous route may be less likely to home and adhere to non-inflammatory cardiac tissue, to remain in the cardiac microenvironment long enough to differentiate and proliferate. The same may even be true for cells injected directly into

coronary artery or into the myocardium itself, although the latter approach may induce local inflammation via the mechanical trauma that occurs. If extrapolated to man, these conclusions raise the possibility that cell transplantation may be a less effective therapy for patients who do not manifest a significant degree of cardiac inflammation, especially those patients with end-stage cardiomyopathy who theoretically would be among those most likely benefit from stem cell therapy.

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CHAPTER 4

Homing of intravenously infused embryonic stem cell-derived cells to injured hearts after myocardial infarction

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ABSTRACT

The present study was designed to test whether intravenously infused embryonic stem cell-derived cells could translocate to injured myocardium after myocardial infarction and improve cardiac function. Cultured embryonic stem cell-derived cells were transfected with green fluorescent protein. Embryonic stem cell-derived cells were administered through the tail vein (approximately 10^7 cells in 1 ml of medium for each rat) every other day for 6 days in 45 rats after myocardial infarction. Six weeks after myocardial infarction and cell infusion, cardiac function, blood flow, and the numeric density of arterioles were measured to test the benefits of cell therapy. An in vitro Transwell assay was performed to evaluate the embryonic stem cell migration. Ventricular function, regional blood flow, and arteriole density were significantly increased in rats receiving intravenously infused embryonic stem cell-derived cells compared with control rats after myocardial infarction. Histologic analysis demonstrated that infused embryonic stem cell-derived cells formed green fluorescent protein-positive grafts in infarcted myocardium. Additionally, positive immunostaining for cardiac troponin I was found in hearts after myocardial infarction receiving embryonic stem cell-derived cell infusion that corresponded to the green fluorescent protein-positive staining. The Transwell migration assay indicated that cultured neonatal rat cardiomyocytes with overexpression of tumor necrosis factor α induced greater migration of embryonic stem cells compared with cardiomyocytes without tumor necrosis factor α expression.

Our data demonstrate that intravenously infused embryonic stem cell-derived cells homed to the infarcted heart, improved cardiac function, and enhanced regional blood flow at 6 weeks after myocardial infarction. The in vitro migration assay suggested that such a homing mechanism could be associated with locally released cytokines, such as tumor necrosis factor α, that are upregulated in the setting of acute myocardial infarction and heart failure.

INTRODUCTION

In recent years, cell transplantation has emerged as a potential therapy for heart failure caused by myocardial infarction (MI) [1-6]. Initial efforts at cellular cardiomyoplasty have transplanted satellite cells [1], skeletal myoblasts [2], bone marrow-derived cells, [5] and fetal cardiomyocytes [6]. Embryonic stem cells (ESCs), which have better plasticity and cardiomyogenic capacity than the cell types listed above, have also been successfully transplanted into infarcted rodent hearts [3] and [4] and the left ventricular (LV) wall of dystrophic mice [7]. Most of the previous studies delivered donor cells through

intramyocardial injection after cardiac surgery. The advantage of this approach is that it traps implanted cells in selected injured areas of the heart. However, the procedure of intramyocardial injection is invasive and might not be suitable for patients with acute MI or severe congestive heart failure. Recently, Chiu and colleagues [8] and [9] demonstrated the feasibility of delivering bone marrow stromal cells with coronary infusion and indicated that marrow stromal cells could traffic through the coronary system into injured myocardium and form cardiomyocytes. Mobilized bone marrow cells, stimulated by stem cell factor or granulocyte colony-stimulating factor, have been shown to repair the infarcted mouse heart and improve ventricular function [10]. It appears that somatic stem cells can migrate to heart tissue and further differentiate into cardiomyocytes [11]. MI is associated with inflammatory responses that include upregulation of mast cells, macrophages, and associated inflammatory cytokines. Experimental MI is also associated with activation of a series of cytokines [12]. Mast cell-derived tumor necrosis factor α (TNF-α) released after myocardial ischemia represents an 'upstream' cytokine responsible for initiating the inflammatory cascade [12]. Released cytokines from injured myocardium might act as chemoattractants for circulating donor cell migration. Our previous study indicated that mouse ESC-derived cells (EDCs) infused intravenously were able to migrate into injured myocardium caused by encephalomyocarditis virus [13] and increased the survival rate of recipient mice. The present study was designed to investigate whether intravenously infused EDCs could translocate to injured myocardium in response to locally released cytokines after MI and improve cardiac function. An in vitro culture system was used for testing whether TNF-α, an inflammatory cytokine that is upregulated in the setting of acute MI and heart failure, could facilitate EDC migration responding to cytokine stimulation.

MATERIALS AND METHODS

EDC preparation and transplantation

The mouse ESC line ES-D3 was purchased from the American Type Culture Collection (Manassas, VA) and cultured with the handing drops method, as previously described [3,4]. Before transplantation, cells dissected from beating clusters were transfected with green fluorescent protein (GFP), a marker for identification of infused cells from host myocardium. Plasmids with an hCMV IE promoter/enhancer-driving GFP gene (5.7 kb) and Gene PORTER transfection reagent were obtained from Gene Therapy Systems Inc. (San Diego, Calif). GFP-labeled EDCs could be detected under fluorescent microscopy at the second day of transfection, and the transfection efficiency was greater than 90% (Fig. 1). Our previous study demonstrated that the action potentials recorded from spontaneously beating EDCs are

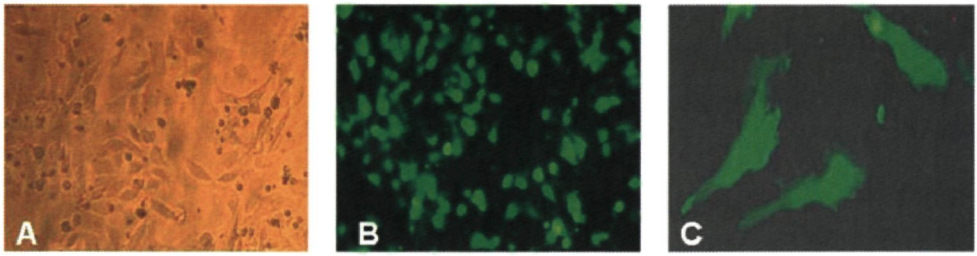

Figure 1. Confluent culture of EDCs under phase-contrast (**A**, original magnification 40x) and fluorescent (**B**, original magnification 40x; **C**, original magnification 400x) microscopy at 3 days after GFP transfection.

very similar to those recorded in neonatal mouse cardiomyocytes [3]. Two days after GFP transfection, cultured EDCs were trypsinized and resuspended in Joklik modified medium (Sigma, St. Louis, MO), with a density of approximately 10^7 cells/ml for cell infusion. Experiments were performed in 45 Wistar male rats (Charles River Laboratories, Wilmington, Mass) aged 3 months. The investigation conformed to the "Guide for the care and use of laboratory animals" published by the US National Institutes of Health (publication no. 85-23, revised 1996), and the protocol was approved by our Institutional Animal Care Committee. MI was created by means of ligation of the left coronary artery during anesthesia with pentobarbital (60 mg/kg administered intraperitoneally), as previously described [3,4]. Approximately 20 minutes after ligation of the coronary artery, EDCs were administered through the tail vein (approximately 10^7 cells in 1 ml of medium for each rat) every other day for 6 days in experimental rats after MI. Control rats after MI received the same operation but were only infused with the equivalent volume of the cell-free medium. The sham group underwent an identical operation with neither ligation of the coronary artery nor cell transplantation.

Measurements of cardiac function and infarct size

Hemodynamic measurements (8 per group) in vivo were performed with a modified method, as described previously [3,4], at 6 weeks after MI and cell infusion. The Millar catheter was carefully advanced into the left ventricle through the carotid artery. The LV systolic pressure (LVSP), the LV end-diastolic pressure, the maximum rate of LV systolic pressure increase ($+dP/dt_{max}$), and the maximum rate of LV systolic pressure decrease ($-dP/dt_{max}$) were recorded on a computer and analyzed by using a PowerLab data-acquisition

system (model ML820, ADInstruments, Colorado Springs, CO) The rat heart was harvested after hemodynamic measurement, weighed, and normalized by body weight Subsequently, the hearts were transversely sectioned into 4 pieces from the apex to the base and prepared for infarct size measurement and histologic study [3,4]

Measurements of regional blood flow and numeric density of arterioles

In another set of animals (7 per group), stable isotope-labeled microspheres (15 μm, BioPAL Inc , Worcester, MA) were used to determine the regional blood flow in anesthetized rats The method was modified from a previous publication [14] In brief, a set of microspheres ($1\ 25x10^6$ in 0 5 ml) was diluted in 0 5 ml of sanSaline saline (BioPAL Inc) and injected into the left atrium over 10 seconds Reference blood samples were withdrawn by using a standard syringe pump (model PHD 2000, Harvard-Apparatus, Holliston, MA) at a constant rate of 2-minute intervals through the femoral artery, resulting in a 2-ml sample used to calculate absolute myocardial blood flow The rat heart was then harvested after achievement of anesthesia The left ventricle was surgically isolated and cut into transmural slices, which were further subdivided into transmural segments Each segment contained approximately equal concentrations of the endocardium and epicardium The average myocardial sample weighed approximately 0 15 g Finally, the tissues and blood samples were shipped to BioPAL Inc for measurement of isotope microspheres and determination of myocardial blood flow In addition, the numeric density of arterioles larger than 20 μm in diameter was counted in each area observed (5 rats for each group and 5 random high-power fields in each rat) on hematoxylin and eosin-stained slides under light microscopy at 400x magnification The number of arterioles in each section was averaged and expressed as the number of arterioles per square millimeter

Histologic study to identify donor cells

The survival of engrafted EDCs was identified by using GFP-positive tissues in paraffin-embedded sections made from rat hearts after MI and cell infusion Transverse sections were also made across the major axis of the liver, lung, kidney, thymus, and spleen in each rat The hearts were sectioned at 5-μm thickness and stained with hematoxylin and eosin Survived infused cells were confirmed by means of identification of GFP expression under fluorescent microscopy and immunostaining for GFP antibody (Abcam, Cambridge, MA) The secondary antibody was anti-rabbit IgG horseradish peroxidase, and the color was produced with diaminobenzidine (DAKO, Carpinteria, CA) Endogenous peroxides were blocked with DAKO peroxide-blocking reagent (DAKO) Rabbit IgG was used as an isotype control New differentiated cardiac tissues from infused EDCs were verified by means of positive

immunostaining of goat anti-cardiac troponin I (cTnI) polyclonal antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, CA). Positive stains were recognized by adding secondary antibodies of chicken anti-goat IgG 594 (Molecular Probes, Eugene, OR). Nuclei were identified by means of 4',6-diamidino-2-phenylindole (DAPI) staining. Nonspecific binding was blocked through incubation with protein-blocking solution (DAKO). We then estimated the area of GFP-positive spots in each sample of myocardial sections (5 rat hearts received EDC infusion) and counted the GFP-positive spots in eight 200x fields that covered the whole heart section. The percentage of surviving cells was calculated by dividing the estimated total area of the left ventricle by the area of GFP-positive spots by using a National Institutes of Health imaging system. The resulting percentage was assumed to be the percentage of surviving cells in the heart that derived from donor stem cells previously infused.

ESC migration assay in vitro

Migration of ESCs was assessed in Transwell plates of 6.5-mm diameter with 5-μm pore filters (Fig. 2A). Mouse embryonic fibroblast feeder cells (STO, American Type Culture Collection) were plated at $3x10^4$ cells/well on fibronectin-coated filters. The adherent cells were cultured for 2 days to obtain confluent STO monolayers. Each lower compartment at the bottom was plated with neonatal rat myocytes. Myocytes in the treated group were transfected with TNF-α cDNA. Cultured neonatal rat myocytes (80% confluence) plated in TI-75 flasks were transfected with TNF-α cDNA (5 μl, $9.9x10^{11}$ particles/ml). The content of TNF-α released by cardiomyocytes into the culture medium was measured with a commercial enzyme-linked immunosorbent assay kit specifically designed for detection of mouse TNF-α (R&D Systems, Inc., Minneapolis, MN; n=7). Before adding ESCs to the upper compartments, STO monolayers were treated with mitomycin-C (10 g/ml) for 90 minutes and washed 3 times with culture medium. GFP-tagged ESCs ($6x10^4$ cells) were added to each upper compartment. Twenty-four hours after incubation, the number of migrated GFP-positive cells in the lower compartments (n=5 runs) was counted during fluorescent microscopy.

Data analysis

All values are presented as means±standard deviation. Data derived from 3 groups in the animal study with repeated measurements were evaluated by means of 1-way analysis of variance (ANOVA). If ANOVA showed a significant difference, an unpaired Student's t-test with the Bonferroni correction was used to compare 2 individual groups. The results of migration assay between 2 individual groups were compared by using the unpaired Student's t-test.

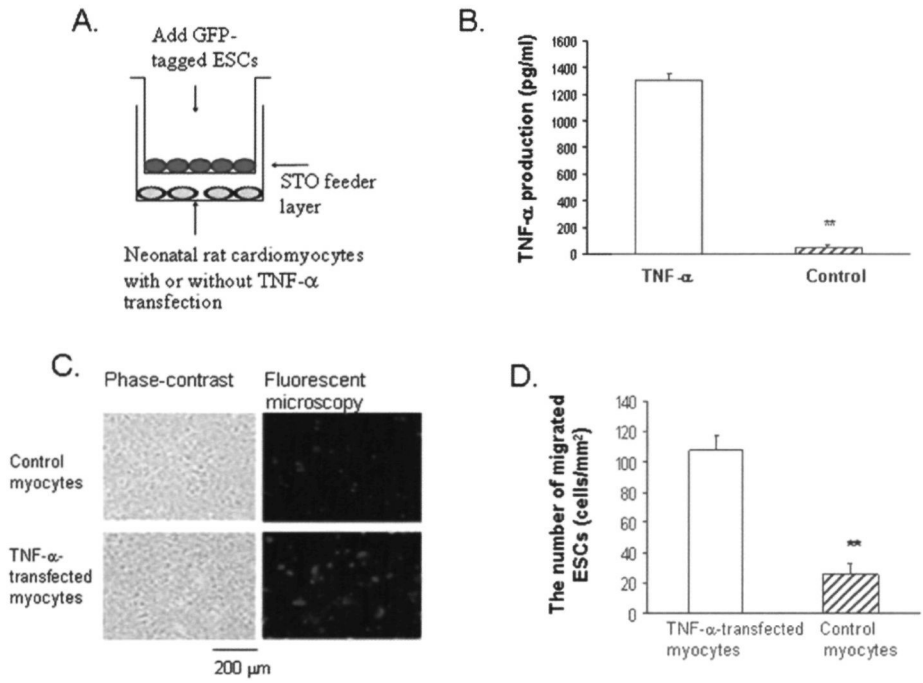

Figure 2. Diagram of embryonic stem cell (ESC) migration assay with Transwell plates (**A**). Each lower compartment of Transwell plates contained one 3-mm glass cover slip plated with neonatal rat myocytes. Green fluorescent protein (GFP)-tagged ESCs were added to the upper compartments of the Transwell dishes and cocultured with myocytes for 24 hours. Migrated GFP-positive cells on each cover slip were counted during fluorescent microscopy for control and tumor necrosis factor α (TNF-α)–transfected myocytes. Efficient production of TNF-α in control and TNF-α transfected myocytes from the lower compartments of the Transwell is shown in panel **B**. Panel **C** demonstrates that more GFP-positive ESCs were detected on the cover slips seeded with TNF-α-transfected myocytes than on those with control myocytes, which indicated that greater ESC migration responded to TNF-α overexpression. The average GFP-positive cells in the lower compartments that were migrated from the upper compartments at 24 hours after culture are shown in panel **D**. TNF-α, The group of TNF-α-transfected neonatal rat myocytes; Control, the group of neonatal rat myocytes without TNF-α transfection. $^{**}P<0.01$, Control group versus TNF-α group.

Table 1. General characteristics of sham-operated rats and rats after MI after EDC infusion

	Sham	MI + control	MI + IV EDCs
BW (g)	432 ± 12	411 ± 15	421 ± 18
HW (g)	1 0 ± 0 1	1.3 ± 0 4	1.2 ± 0 3
HW/BW100	0.23 ± 0 03	0.32 ± 0.04	0.28 ± 0.03†
HR (beats/min)	390 ± 26	402 ± 27	411 ± 30

Values are presented as means±standard deviation Measurements were conducted at 6 weeks after embryonic stem cell-derived cell or medium infusion in 8 rats for each group. Sham, Sham-operated rats; MI+control, myocardial infarction rats with cell-free medium infusion; MI+IV EDCs, myocardial infarction rats with embryonic stem cell-derived cell infusion; BW, body weight; HW, heart weight, HW/BW, ratio of heart weight/body weight; HR, heart rate. $P<0.01$ versus sham group; †$P<0.05$, MI+IV EDC group versus MI+control group

RESULTS

Improvement of cardiac function and blood perfusion after intravenous infusion of EDCs

Six weeks after intravenous infusion of EDCs, the ratio of heart weight to body weight was significantly increased in the MI control and MI-EDC infusion groups compared with that seen in the sham-operated group. Cell therapy in rats after MI partially attenuated not only the severity of cardiac hypertrophy but also the infarcted area versus that seen in control rats after MI (Table 1). Additionally, the control rats after MI had a lower LVSP, lower +dP/d$tmax$, lower -dP/dt_{max}, and higher LV end-diastolic pressure compared with values in the sham and MI cell-treated groups (Fig. 3). EDC infusion at 6 weeks after MI significantly improved cardiac function, reflected by an increase in LVSP, +dP/dt_{max}, and -dP/dt_{max} (Fig. 3). Additionally, the regional blood flow assessed by isotope microspheres and the arteriole density were significantly decreased in the rats after MI (Fig. 4). EDC infusion significantly increased the LV myocardial blood perfusion and the numeric density of arterioles compared with that seen in the MI control group.

Histologic study and identification of infused EDCs

Hematoxylin and eosin staining of heart sections from rats after MI receiving cell therapy showed less necrosis in infarcted and surrounding areas at 6 weeks after cell infusion (Fig. 5). Significant fibrosis was found in postinfarcted myocardium in the control hearts after MI without cell infusion. Paraffin-embedded sections from rat hearts after MI at 6 weeks after cell treatment showed GFP-positive spots under fluorescent microscopy that were further

stained positively with GFP antibody (Fig. 6). In contrast, sections from sham-operated hearts and control hearts after MI had no such GFP-positive tissue stained with GFP antibody. Positive immunostaining for cTnI was found in EDC-infused hearts after MI that corresponded to GFP-positive spots (Fig. 7). These data suggest that infused EDCs not only survived in injured myocardium but also differentiated into cardiac tissue. In addition, GFP-positive cells were not identified outside the infarcted hearts, except in the spleen, which contained some GFP-positive areas but stained negative to troponin I (data not shown). The surviving cells examined in a cross-section of the heart were identified as expressing GFP in the group of rats after MI receiving cell infusion. The average percentage of GFP-positive cells was 5.3% of the total left ventricle.

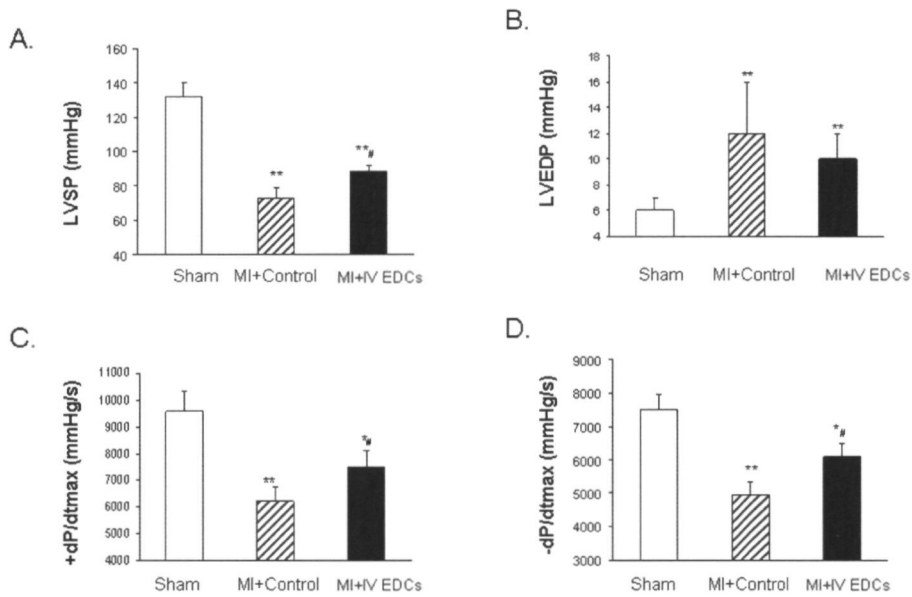

Figure 3. Cardiac function assessed with a Millar catheter in experimental rats at 6 weeks after myocardial infarction (MI) and embryonic stem cell-derived cell (EDC) infusion through tail vein injection. (**A**) Left ventricular systolic pressure (LVSP); (**B**) left ventricular end-diastolic pressure (LVEDP); (**C**) maximum rate of peak left ventricular systolic pressure increase ($+dP/dt_{max}$); (**D**) maximum rate of peak left ventricular systolic pressure decrease ($-dP/dt_{max}$). Sham, Sham-operated rats; MI+Control, rats after with cell-free medium infusion; MI+IV EDCs, rats after MI with EDC infusion ($n = 8$ in each group). *P<0.05 and **P<0.01 versus sham group; #P<0.05 versus MI+Control group.

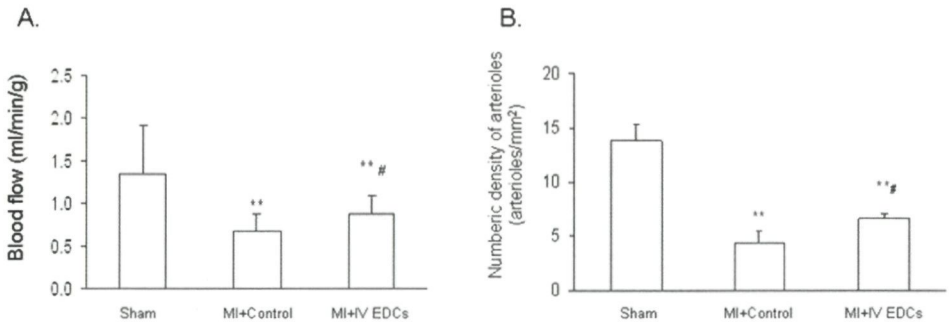

Figure 4. Left ventricular blood flow measured with isotope microspheres is shown in panel **A** (7 for each group). Numeric densities of arterioles are shown in panel **B** (8 for each group). The results indicated that intravenously infused embryonic stem cell–derived cells (EDCs) significantly improved regional blood perfusion and enhanced angiogenesis at 6 weeks after myocardial infarction (MI) and EDC treatment. Sham, Sham-operated rats; MI+Control, rats after MI with cell-free medium infusion; MI+IV EDCs, rats after MI with EDC infusion. $**P<0.01$ versus sham group; $\#P<0.05$ versus MI+Control group.

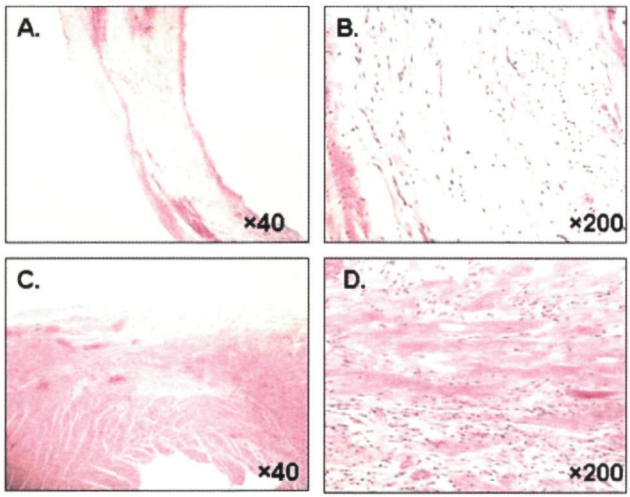

Figure 5. Representative rat myocardium stained with hematoxylin and eosin were from a rat at 6 weeks after myocardial infarction (MI) with cell-free medium infusion (**A** and **B**) and a rat at 6 weeks after MI and embryonic stem cell-derived cell (EDC) tail vein infusion (**C** and **D**). Cell grafts were clearly found within the infarcted zone in infracted myocardium after EDC infusion. In contrast, the tissue in the infarcted regions of the control myocardium appeared fibrotic and relatively acellular.

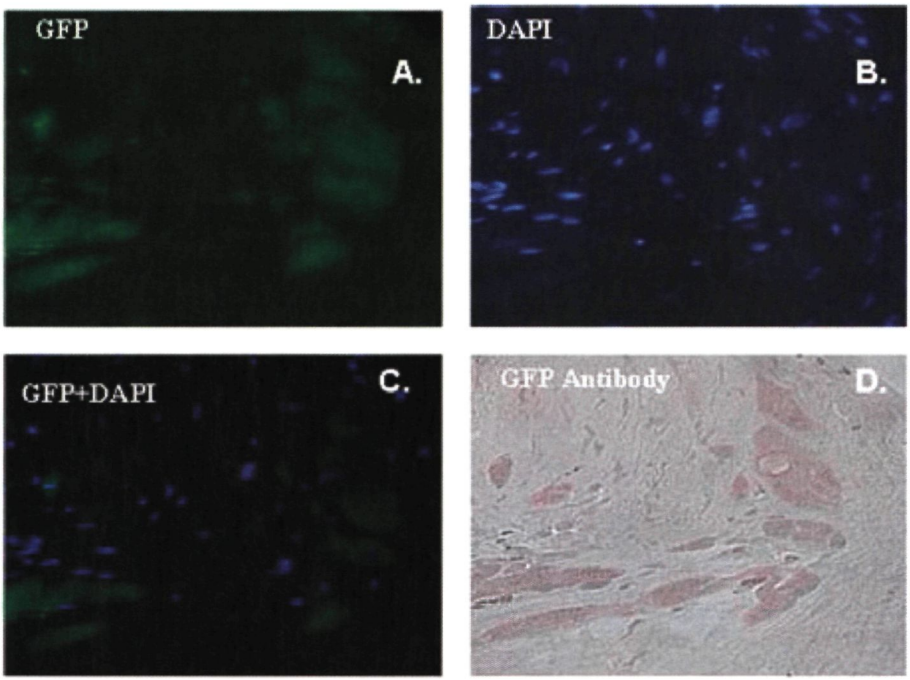

Figure 6. Infused embryonic stem cell-derived cells (EDCs) in postinfarcted rat myocardium were identified at 6 weeks after myocardial infarction (MI) and tail vein injection. Infused cells were clearly seen in the infarcted area with green fluorescent protein (GFP) fluorescence (**A**) and further verified positive to GFP antibodies (**D**), which demonstrated that circulating infused EDCs could home to injured myocardium.

In vitro assay of TNF-α-induced ESC migration

The lower compartments of the Transwell plates were cultured with neonatal rat cardiomyocytes with or without transfection of TNF-α cDNA. The amount of TNF-α production was significantly increased in myocytes transfected with TNF-α compared with that from the control myocytes in culture medium of the lower compartments (Fig. 2B; n=7 runs for each). Figure 2C and D (n=5 runs for each), demonstrate significantly greater ESC migration in response to TNF-α incubated for 24 hours after adding GFP-tagged ESCs to the upper compartments. These data suggest that cultured neonatal rat cardiomyocytes with overexpression of TNF-α attract more ESCs migrating from the upper compartment to the lower compartment.

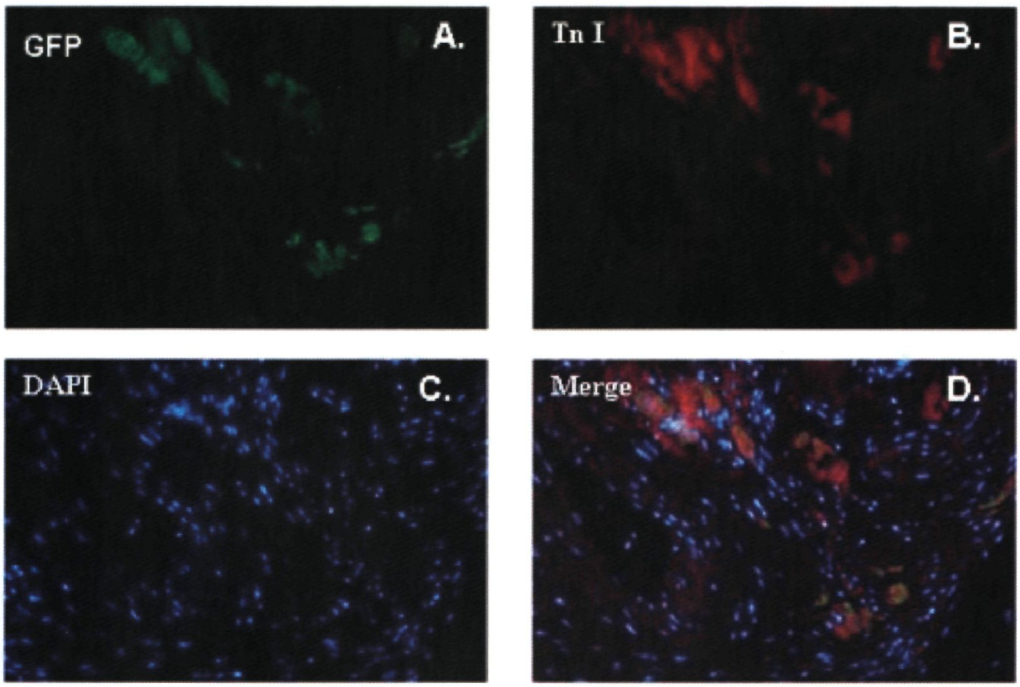

Figure 7. Myocardial sections from postinfarcted myocardium 6 weeks after embryonic stem cell-derived cell (EDC) infusion. Infused green fluorescent protein (GFP)-positive EDCs (**A**) homing into the infarcted area were stained positive to cardiac troponin I (TnI; **B**). DAPI showed nuclear staining (**C**). The merge (**D**) of GFP, cardiac TnI, and nucleus of cells demonstrated that infused GFP-labeled EDCs could not only home to the infarcted zone but also differentiate into cardiac tissue. (Original magnification 200x). Panel **B** shows the nuclear staining with DAPI, and panel **C** shows the merge of GFP and DAPI staining (Original magnification 200x).

DISCUSSION

Cell transplantation has emerged as a potential therapy to treat cardiac dysfunction resulting from MI. Various cell types appear to be promising candidates because of their ability to integrate into the host heart tissue [1-6,15,16] and improve cardiac function [1-6,17] Differentiation efficiency of bone marrow-derived stem cells into adult cardiomyocytes appears limited [18]. ESCs, on the other hand, are pluripotent cells derived from the early embryo and retain the ability to differentiate into all cell types, including cardiomyocytes

[19,20] The availability of human ESCs and the technique of development for enrichment of cardiomyocytes derived from human ESCs have paved a possible way to use ESCs for a therapeutic approach for animal study and clinical application Our previous studies [3,4] demonstrated that ESCs could be implanted into rat myocardium with intramyocardial injection after MI The viability and regeneration capacity of engrafted ESCs were demonstrated by their positive immunostaining for α-myosin heavy chain and cTnI Studies have also shown the feasibility of transcoronary infusion of donor cells into the intact rat heart [21], doxorubicin-induced failing rat heart [22] and infarcted rat heart [9] However, the clinical situation during the early stage of MI might be suitable neither for invasive cardiothoracic surgery with intramyocardial injection of cells nor for relatively less invasive interventional catheterization Such surgical or invasive procedures might be associated with high mortality in critically ill patients Moreover, intracoronary cell transplantation has an apparent disadvantage because of the risk of coronary embolism [21]

Recent studies demonstrated that intravenously infused donor cells can migrate to an injury site [23,24] and induce angiogenesis [24] in a rat model of stroke It has been shown that an animal model of MI [25] is associated with inflammatory infiltration, which is abundant on days 2 to 3 but gradually decreases and disappears on day 7 after MI Our present study indicates that intravenously administrated EDCs can traffic through the circulation to postinfarcted myocardium and differentiate into cardiac tissue The mechanism might be associated with the inflammatory response after MI, and locally released cytokines might facilitate infused EDC migration toward injured myocardium

Orlic and associates [10] reported that subcutaneous injection of stem cell factor or granulocyte colony-stimulating factor facilitated translocation of native bone marrow cells into infarcted myocardium, resulting in a significant degree of tissue differentiation 27 days later However, recruitment of endogenous adult stem cells after ischemic injury might not be enough to achieve functional improvement Our data demonstrated that intravenously infused EDCs can translocate to injured myocardium through the circulation No significant numbers of GFP-positive donor cells were found in noninjured organs, including the lung, kidney, and liver, except for the spleen, in which a few GFP-positive spots were found (data not shown) Trapped EDCs might infiltrate through vessels into injured myocardium Within a suitable surrounding niche, infused EDCs differentiated into new cardiac-like tissue to replace dead or damaged myocardium, which was supported by positive staining to cTnI in the grafts Moreover, cardiac protective factors (e g , vascular growth factor) might be released from infused EDCs, which not only rescue damaged cardiac tissue but also promote angiogenesis, as reflected by the enhancement of regional blood perfusion and increased numbers of arterioles observed in the present study Therefore the beneficial effects on improvement of cardiac function and the reduction of infarct size after EDC infusion might result from the

synergistic effects of myogenesis and angiogenesis. Other factors, such as the antiapoptotic role of engrafted stem cells, might also partially contribute to the functional benefits. More experimental studies are needed to address the mechanisms of cell transplantation therapy.

The homing response of intravenously infused EDCs might be associated with the response to cytokines that are released locally in injured myocardium. TNF-α, a member of the proinflammatory cytokines, is considered to participate in the interactive signal-transduction cascade that regulates inflammatory and immunologic responses and triggers leukocyte infiltration through its chemotactic properties [26]. After MI, there is an immediate and rapid upregulation of TNF-α in the myocardium as part of the immune system response in the rat MI model [27]. Furthermore, detectable expression of TNF-α persisted to day 35 after MI. Levels of TNF-α protein in the infarct and peri-infarct zones increased early to 8- to 10-fold above normal levels and increased to 4- to 5-fold in the contralateral zone [27]. Thus it is reasonable to use TNF-α as a representative factor in the cytokine family to test whether cytokines released from local injured myocardium after MI facilitate migration of infused EDCs. During fluorescent microscopy, we detected more GFP-positive cells on the cover slips seeded with TNF-α-transfected cardiomyocytes than on cover slips plated with control cardiomyocytes. Cardiomyocytes with overexpression of TNF-α attracted more ESCs, which migrated from one compartment to another in response to TNF-α stimulation. This finding suggests that cytokines produced by injured myocardium after acute MI perform a major role as chemoattractants for intravenously infused EDCs and result in infused cells migrating into sites of injury. Using a Dunn chamber (a direct viewing chemotaxis chamber with concentric wells), we recently showed that ESCs are highly motile and respond to different concentrations of TNF-α in a dose-related manner [28]. In addition, our previous study [29] indicated that the molecular pathway of stem cell migration stimulated by excessive TNF-α is through the type II TNF-α receptor. Activation of p38 and JNK is required for TNF-α-enhanced stem cell migration.

There are several limitations to the present study. Interpreting the functional benefits of EDC infusion in rats after MI should be done cautiously because cardiac function was measured in unloaded experimental condition and different loading conditions might affect functional results measured with a Millar catheter. A previous study [30] demonstrated that intravenously infused bone marrow-derived mesenchymal stem cells at 1 week after infusion were trapped either in the infarcted or border zone and also lodged in the lung, liver, spleen, and bone marrow. However, the present study indicated that infused donor cells were identified only in the infarcted heart and the spleen at 6 weeks after MI and cell infusion. These differential results might be related to the different time points chosen to track the fate of infused donor cells among various studies.

In conclusion, the present study indicates that intravenously infused EDCs can traffic through the circulation into injured myocardium. The migration of infused EDCs might be associated with local cytokine release after acute MI. EDCs homing to injured myocardium because of chemotactic properties of inflammatory cytokines released locally could differentiate into cardiac tissue and improve ventricular function. The functional benefits are also related to enhancement of regional blood perfusion in infarcted myocardium after EDC infusion. The present strategy simplifies the pathway for cell delivery by using intravenous infusion. This approach could potentially be used in either severe heart failure or during the acute phase of MI in a critically ill patient who might not tolerate cell transplantation through surgical operation or invasive catheterization.

ACKNOWLEDGEMENTS

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CHAPTER 5

Antiapoptotic effect of implanted embryonic stem cell-derived early-differentiated cells in aging rats after myocardial infarction

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ABSTRACT

This study tested whether implanted embryonic stem cell-derived early-differentiated cells (EDCs) lead to improvement in cardiac function by preventing cardiac apoptosis in aging rats after myocardial infarction. Cardiac apoptosis after transplantation of EDCs was assessed in situ by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling reaction (TUNEL) staining as well as by measurements of protein levels of cleaved caspases 3, Bax and Bcl-2. Our results indicate that cell transplantation improved cardiac function at 6-months observation. The frequency of apoptotic cells in the peri-infarcted myocardium 3 days after cell transplantation was significantly decreased in the cell transplantation group. EDC therapy decreased the protein levels of cleaved caspase 3 and Bax, and increased the level of Bcl-2 in comparison to myocardial infarction control. Additionally, the number of apoptotic cells decreased significantly in cardiomyocytes precocultured with EDCs. This study demonstrates that functional improvement of EDC transplantation may at least in part be related to a reduction in cardiomyocyte apoptosis.

INTRODUCTION

Advanced age, even in healthy individuals without apparent cardiac disease, is associated with changes in heart structure and compromised cardiac reserve [1,2]. Congestive heart failure, caused primarily by myocardial infarction (MI), is the major cause of hospitalization for people older than 65 years [3]. Despite treatment strategies developed in the past few decades that were aimed at different pathophysiological mechanisms of myocardial disease, morbidity and mortality due to cardiac dysfunction after MI remains a clinical challenge. Aging decreases the functional reserve of the heart that is associated with the loss of cardiomyocytes due to the progressive process of apoptosis. Previous studies [4,5] demonstrated that there is an increase in the number of apoptotic myocytes after MI. Among the treatment strategies available, the maintenance of contractile mass, determined by the number of functional cardiomyocytes, is a major goal in the therapy of heart disease. Inhibition of myocyte apoptosis could prevent the loss of contractile cells and thus provide a new approach to cardiac dysfunction in the aging heart after MI.

In recent years, cell transplantation has emerged as a potential therapeutic approach for repairing damaged myocardium. Our previous studies [6,7] and those of others [8-11] have demonstrated that engrafted stem cells can survive and differentiate into functional cardiac tissue in an animal model of infarction. However, the mechanisms of cell therapy have not

been fully elucidated The transdifferentiation ability of hematopoietic stem cells has been challenged [12-14], and an intense debate over cell fusion versus transdifferentiation has resulted [15] Our previous studies indicate that newly regenerated cardiomyocytes after early-differentiated cell (EDC) transplantation compose approximately 5 3–7 3% of the total left ventricle (LV) [6,16] Cell fusion appears to be an infrequent event, occurring perhaps once in 10,000–100,000 cells [17] Thus, the small percentage of stem cell–derived cardiomyocytes and the low frequency of cell fusion cannot explain the significant improvement of cardiac function after stem cell transplantation One potential mechanism by which stem cells might lead to improvement in cardiac function in the setting of MI is by preventing apoptosis of cardiomyocytes The present study was designed to test the hypothesis that EDCs prevent apoptosis of cardiomyocytes and lead to functional improvement after local implantation in aging rats in the setting of MI

MATERIALS AND METHODS

EDC preparation, experimental MI and EDC transplantation

The mouse embryonic stem cells (ESC) line, ES-D3, was purchased from the American Type Culture Collection (ATCC, Manassas, VA), and cultured using a method previously described [6,7] Cultured EDCs were finally trypsinized and resuspended in Joklik's modified medium (Sigma, St Louis, MO) with a density of $2x10^7$ cells/ml for cell transplantation Experiments were performed in a total of 80 senescent male Fischer 344 rats aged 24 months (obtained from the National Institute on Aging, Bethesda, MD) 1 week after arrival The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the U S National Institutes of Health (NIH Publication No 85-23, revised 1996), and the protocol was approved by our Institutional Animal Care Committee Rats were anesthetized with pentobarbital sodium (60 mg/kg) by intraperitoneal injection, and were ventilated by using a small animal ventilator (Harvard Apparatus, South Natick, MA) MI was induced by ligation of the left anterior descending coronary artery and verified by observing blanching of the myocardium distal to the ligation [6,7] Intramyocardial injection of the EDC suspension (50 μl, 10^6 cells) was performed in three different sites in the LV, one within the infarct area and two in the myocardium bordering the infarct area MI-Control rats received the same volume of the cell-free medium as the rats receiving EDC transplantation The Sham group underwent an identical surgery with neither ligation of the coronary artery nor cell transplantation The study comprised two experimental cohorts a subgroup of aged rats (15 in

Sham, 20 in MI-Control, and 20 in MI-EDC) was followed-up for 6 months after MI to determine the long-term functional benefit of EDC transplantation. Another cohort of aged rats (7 rats for each group) was observed for 3 days after MI to determine the antiapoptotic role of implanted EDCs. Furthermore, an in vitro apoptotic assay in cardiomyocytes precocultured with EDCs under conditions of hypoxia was applied to verify the antiapoptotic role of EDCs.

Measurements of cardiac function and infarct size

Hemodynamic measurements in vivo were performed with a method described previously [6,7] in pentobarbital-anesthetized rats 6 months after EDC transplantation. Briefly, a carotid artery was isolated and cannulated with a 3-Fr high-fidelity Mikro-tip catheter connected to a pressure transducer (Millar Instruments, Houston, TX). The Millar catheter was carefully advanced into the LV. The LV systolic pressure (LVSP), the LV end-diastolic pressure (LVEDP), the maximum rate of LV systolic pressure rise ($+dP/dt_{max}$), and the maximum rate of LV systolic pressure fall ($-dP/dt_{max}$) were recorded on a computer and analyzed by a PowerLab data-acquisition system (model ML820; ADInstruments, Colorado Springs, CO). The rat heart was harvested after hemodynamic measurement under deep anesthesia with pentobarbital (100 mg/kg). Subsequently, the hearts were transversely sectioned into 4 pieces from the apex to the base, and were prepared for hematoxylin and eosin staining. Infarct size was calculated by dividing the sum of the planimetered endocardial and epicardial circumferences of the infarcted area by the sum of the total epicardial and endocardial circumferences of the LV [6,7].

Assessments of cardiac apoptosis in situ and the Western blot

In another cohort of aging rats (n=38), cardiac apoptosis in situ was assessed by examination of morphological features under light microscopy and by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling reaction (TUNEL) labeling of the 3'OH ends of DNA in paraffin-embedded heart sections 3 days after MI and EDC transplantation. The hearts were harvested from MI rats after measurement of hemodynamics, fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μm. Following incubation with terminal deoxynucleotidyl transferase (TdT) enzyme, apoptotic nuclei (brown) were identified by staining with DAB (ApoTag plus peroxidase in situ Apoptosis detection kit; Chemicon, Temecula, CA). Nonapoptotic nuclei (blue) were identified by staining with 0.5% methyl green.

The protein levels of Bax, Bcl-2, and cleaved caspase 3 were measured in aged rat ventricles from Sham, MI-Control, and MI-EDC rats 3 days after acute surgery. Briefly,

frozen tissues were homogenized in RIPA buffer containing protein inhibitor on ice and then sonicated Protein concentrations were determined with a modified Bradford reaction (Bio-Rad, Hercules, CA) and then equal amounts of total protein were separated on a precast sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel (Bio-Rad) Separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane blocked in 5% (wt/vol) nonfat dry milk in 0 1% Tris-buffered saline with tween (TBST) After incubation with primary antibodies (1 1000 dilution of Bax monoclonal antibody obtained from Cell Signaling Technology, Inc [Danvers, MA], 1 400 dilution of Bcl-2 monoclonal antibody obtained from Santa Cruz Biotechnology, Inc [Santa Cruz, CA), or 1 1000 dilution of cleaved Caspase 3 antibody obtained from Cell Signaling Technology, separately) and the second antibodies (Santa Cruz Biotechnology), the membranes were visualized with enhanced chemiluminescence (ECL) plus (Amersham Bioscience, Piscataway, NJ) and exposed to Kodak MR films The relative amounts of Bax, Bcl-2, and cleaved caspase 3 were determined densitometrically using a NIH image system, and the protein levels of GAPDH were used as an internal control

Apoptotic assay in vitro with isolated cardiac myocytes cocultured with EDCs

There is no method available to culture aging cardiomyocytes Therefore, we performed an in vitro apoptotic assay by using adult cardiomyocytes that were isolated by enzymatic dissociation to further determine the antiapoptotic effect of EDCs in vitro Briefly, 3-month-old adult rat hearts were harvested under pentobarbital anesthesia and then perfused for 3 minutes with Ca^{2+}-free Krebs–Henseleit buffer containing (in mmol/l) 118 NaCl, 4 7 KCl, 1 2 KH_2PO_4, 1 2 $MgSO_4$, 25 $NaHCO_3$, 25 HEPES, pH 7 4 at room temperature The perfusate was then switched to an enzyme solution containing collagenase-II at 0 3 mg/ml (Worthington type II at 266 U/mg) and hyaluronidase at 0 3 mg/ml (Sigma type II at 667 U/mg) for another 20 minutes Ventricular tissue was then finely minced and shaken gently in enzyme solution containing trypsin and DNase I for another 20 minutes Isolated cardiac myocytes were filtered through a nylon mesh, and resuspended in Medium-199 containing albumin at 2 mg/mL, carnitine at 0 4 mg/ml, creatine at 0 66 mg/ml, taurine at 0 62 mg/ml, insulin at 2 μL/mL, and penicillin/streptomycin at 100 U/ml Thereafter, myocytes isolated from each experimental rat were plated onto laminin-precoated culture plates consisting of three groups for hypoxia–apoptosis assay normoxic cardiomyocytes, hypoxic cardiomyocytes, and hypoxic cardiomyocytes precocultured for 12 hours with EDCs Each group consisted of five rats Experimental cardiomyocytes were placed in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) containing 0 5% of oxygen for 12 hours The oxygen level in the chamber was monitored with an oxygen analyzer (Vascular Technology Inc , Nashua, NH)

Annexin-V (10 µl/ml medium; Calbiochem, San Diego, CA) was added to each plate to detect apoptotic cardiomyocytes. Ten randomly selected fields of cultured cardiomyocytes isolated from each experimental rat were counted for green fluorescent cells and total cardiomyocytes under Nikon videomicroscopy (Eclipse TE2000-U). Image capture and analysis were performed by using IPLab 3.6 software (Scanalytics Inc., Fairfax, VA). The number of Annexin-V-labeled cells was normalized to the total number of cells as counted by phase-contrast microscopy of the same field.

Data analysis

All values are presented as mean±standard deviation. Data derived from three groups (Sham, MI-Control, and MI-EDCs) were evaluated by using analysis of variance (ANOVA) with repeated measurements. Differences between two groups were compared by using the unpaired Student's *t*-test with Bonferroni's correction. Survival during the 6-month observation period was analyzed by standard Kaplan–Meier analysis, and a statistical comparison among curves was made with the log-rank test. A level of $P<0.05$ was considered significant.

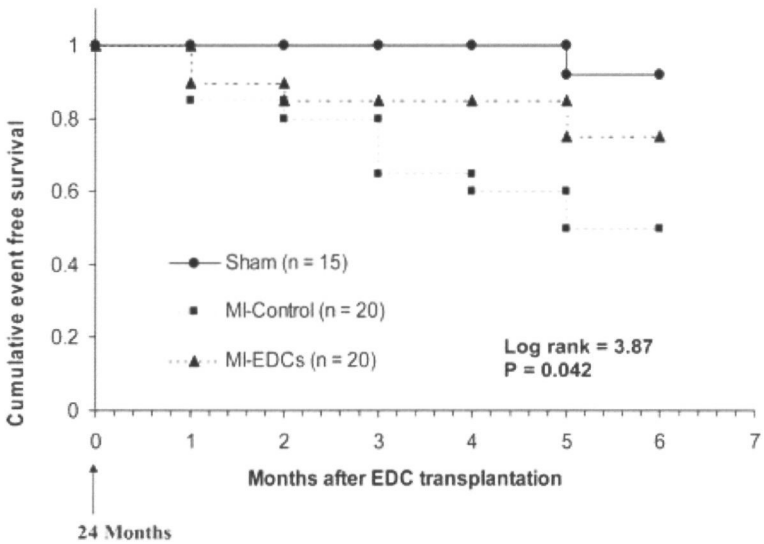

Figure 1. Kaplan–Meier survival curves during 6-month observation of Sham rats, myocardial infarction (MI)-Control rats without cell therapy, and rats receiving early-differentiated cell (EDC) transplantation (MI-EDC). Compared with the MI-Control group, stem cell transplantation significantly increased survival.

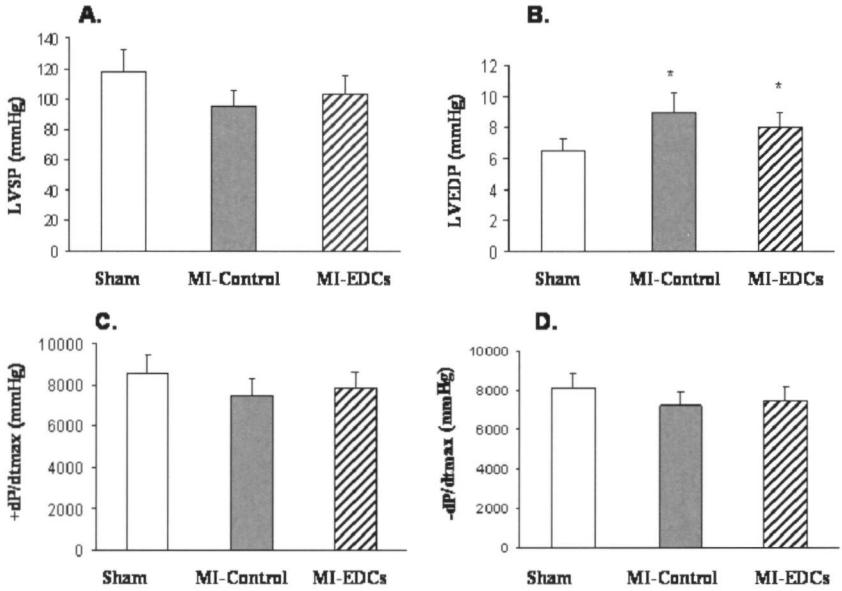

Figure 2. Cardiac function assessed by a Millar catheter in aging rats at 3 days after myocardial infarction (MI) and early-differentiated cell (EDC) transplantation. (**A**) Left ventricular systolic pressure (LVSP); (**B**) left ventricular end-diastolic pressure (LVEDP); (**C**) maximum rate of peak left ventricular systolic pressure rise ($+dP/dt_{max}$); (**D**) maximum rate of peak left ventricular systolic pressure fall ($-dP/dt_{max}$). Sham (n=7), sham-operated rats; MI-Control (n=7), MI rats with cell-free medium injection; MI-EDCs (n=8), MI rats with EDC injection. *P<0.05 vs Sham.

RESULTS

Measurement of cardiac function after EDC transplantation

Approximately 15% of rats (from a total of 80) died within the first day after surgery. In the surviving rats, the 6-month survival rate evaluated by Kaplan–Meier analysis was significantly increased in the group of experimental rats that received stem cell transplantation in comparison to MI-Control rats (Fig. 1). Cardiac function assessed by hemodynamics was measured at two different time points (3 days and 6 months) after MI and stem cell transplantation. After 3 days of MI, ventricular function significantly decreased in the groups of MI rats as compared to age-matched Sham rats (Fig. 2). The decrease was mainly reflected by a reduction in diastolic function (i.e. LVEDP). Three days of EDC treatment did not provide significant functional benefit to the aging hearts after MI. However, our data indicate

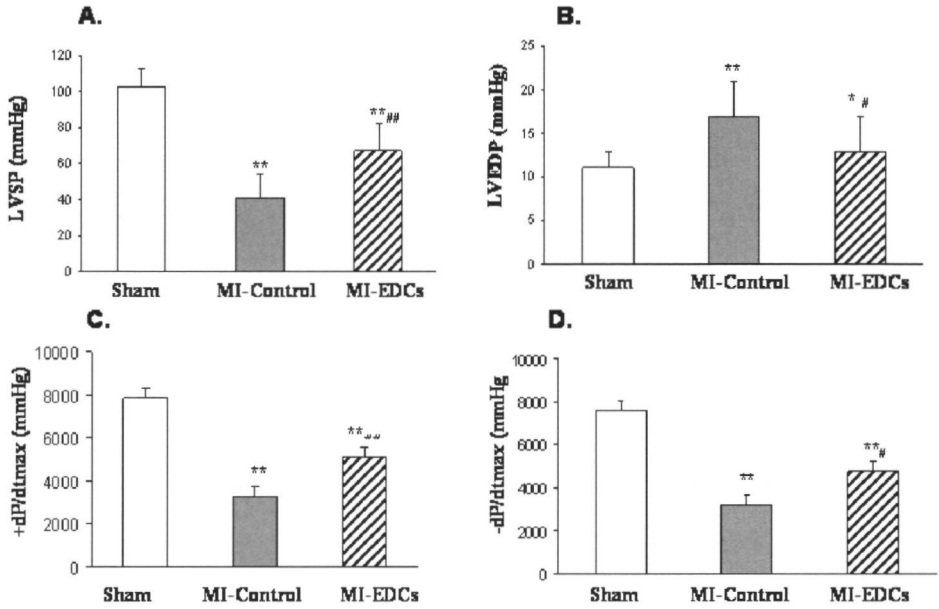

Figure 3. Cardiac function assessed by a Millar catheter in aging rats at 6 months after myocardial infarction (MI) and early-differentiated cell (EDC) transplantation. (**A**) Left ventricular systolic pressure (LVSP); (**B**) left ventricular end-diastolic pressure (LVEDP); (**C**) maximum rate of peak left ventricular systolic pressure rise ($+dP/dt_{max}$); (**D**) maximum rate of peak left ventricular systolic pressure fall ($-dP/dt_{max}$). Sham (n=8), sham-operated rats; MI-Control (n=10), MI rats with cell-free medium injection; MI-EDCs (n=10), MI rats with EDC injection. *P<0.05, **P<0.05 vs Sham; $^{\#}P$<0.05, $^{\#\#}P$<0.01 vs MI-Control.

that stem cell transplantation significantly improved cardiac function at 6 months after MI and EDC transplantation. This improvement was reflected by an increase of LVSP, $+dP/dt$, and $-dP/dt$, as well as a decrease in LVEDP (Fig. 3) compared to these measurements in the MI-Control rats that received cell-free medium only. Additionally, infarct size was significantly reduced in the aging MI rats compared to the MI-Control rats 6 months after EDC transplantation (36±3% in MI-EDCs vs 42±4% in MI-Control, P<0.05).

Cardiac apoptosis in infarcted myocardium and the Western blot

Apoptotic cardiomyocytes in the peri-ischemic areas from Sham, MI-Control, and MI-EDC rats 3 days after surgery (5 hearts for each group) were detected by in situ TUNEL

staining. High-power microscopy (x200) demonstrated that TUNEL-positive staining was localized primarily in the nuclei of cardiomyocytes. There were rare TUNEL-positive nuclei in Sham aging hearts. The TUNEL-positive cells increased at 3 days after MI in the peri-ischemic myocardium (Fig. 4). Additionally, the TUNEL-positive myocytes in the peri-infarction area were counted and normalized to 100 total nuclei in the same sections (Fig. 4). The number of TUNEL-positive myocytes was significantly increased in the infarcted hearts 3 days after MI, whereas EDC transplantation significantly reduced apoptotic cardiomyocytes in comparison to the MI-Control myocardia.

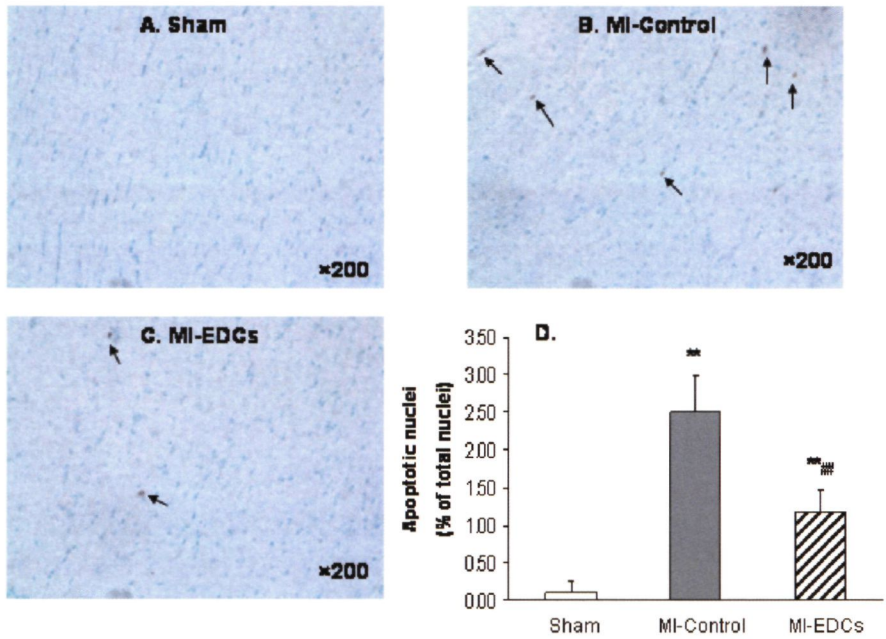

Figure 4. Apoptosis in peri-infarcted myocardium by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling reaction (TUNEL) staining from the groups of sham-operated (Sham, **A**), myocardial infarcted rats with cell-free medium injection (MI-Control, **B**), and MI rats with early-differentiated cell (EDC) injection (MI-EDC, **C**). Apoptotic nuclei (arrows) and nonapoptotic nuclei turned out brown and blue, respectively, by TUNEL staining. Normalized apoptotic nuclei were are indicated in (**D**) as the mean number of TUNEL-positive nuclei per 100 total nuclei in the peri-infarcted myocardium at 3 days after MI. EDC transplantation significantly decreased the frequency of apoptotic nuclei. (Magnification x200; 5 hearts per group.) $^{**}P<0.01$ vs Sham; $^{\#\#}P<0.01$ vs MI-Control.

To elucidate the molecular mechanisms involved in the antiapoptotic effect of EDC therapy, we measured protein levels of Bcl-2, Bax, and cleaved caspase 3 in additional LVs from Sham, MI-Control, and MI-EDC rats (5 for each group) at 3 days after MI surgery. Levels of measured proteins were analyzed as a ratio compared to GAPDH. As shown in Figure 5, there was a significant increase of cleaved caspase 3 in MI rat hearts. The protein level of cleaved caspase 3 was reduced in rat hearts at 3 days after MI and EDC transplantation. The level of Bax increased in the MI hearts compared to the Sham hearts at 3 days after MI operation. EDC treatment at 3 days after cell transplantation significantly decreased the expression of Bax. In contrast, the protein levels of Bcl-2 increased significantly in the rat hearts that received EDC transplantation as compared to the MI-Control hearts.

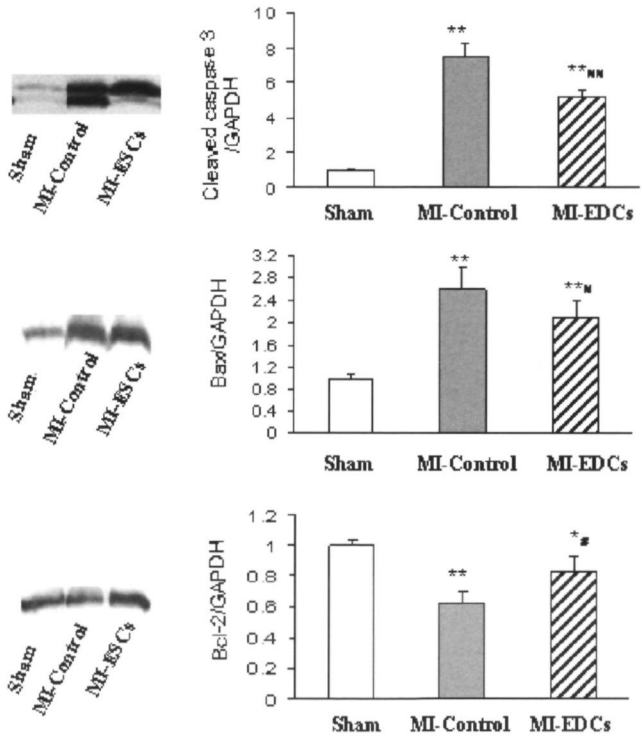

Figure 5. Protein levels of cleaved caspase 3, Bax, and Bcl-2 measured in sham-operated (Sham) and infarcted aging hearts 3 days after surgery (5 for each group). Left traces: Western blots; Right traces: densitometric analysis of measured protein/GAPDH. *P<0.05, **P<0.01 vs Sham; $^{\#}P$<0.05, $^{\#\#}P$<0.01 vs myocardial infarcted rats with cell-free medium injection (MI-Control). MI-EDC=MI rats with early-differentiated cell injection.

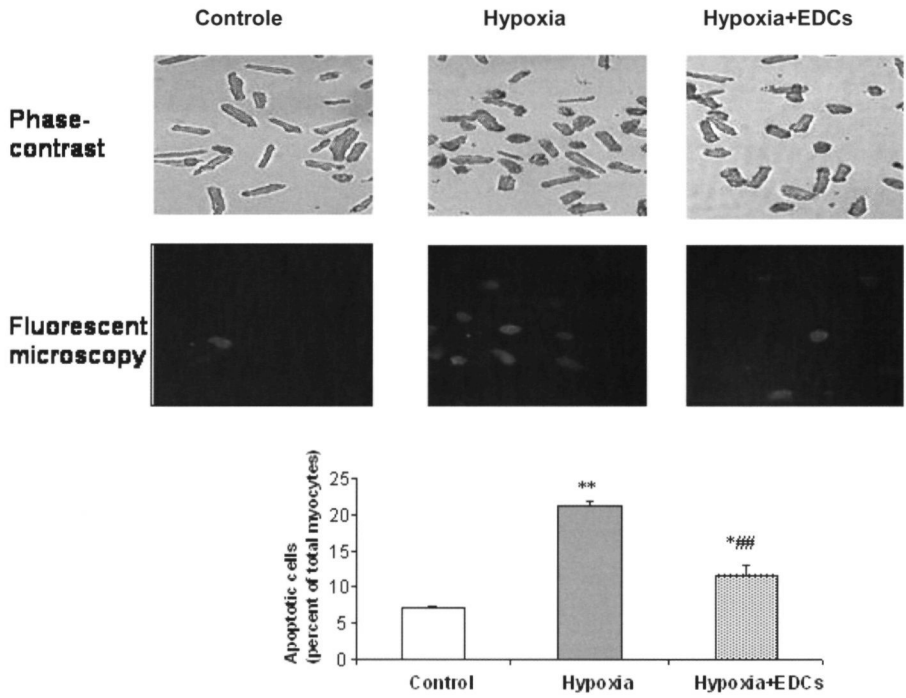

Figure 6. Cellular viability was determined in cultured cardiomyocytes 12 hours after hypoxia. Ten randomly selected fields of cultured cardiomyocytes isolated from each experimental rat were counted to obtain an average value of cardiomyocytes. Each study group consisted of five rats. Positive staining to Annexin-V indicates apoptotic cardiomyocytes. Data show a significant increase of apoptotic cells after 12-hour hypoxia compared to the control group that was under normoxic conditions. However, the number of apoptotic cells significantly decreased in cardiomyocytes precocultured for 12 hours with early-differentiated cells (EDCs). Control = Cardiomyocytes cultured under normoxia; Hypoxia = cardiomyocytes cultured under condition of hypoxia; Hypoxia-EDCs = cardiomyocytes precocultured for 12 hours with EDCs under hypoxic conditions. $^{*}P<0.05$, $^{**}P<0.01$ vs Control; $^{\#\#}P<0.01$ vs Hypoxia.

Assessments of cardiac apoptosis on cultured cardiomyocytes

We next examined the antiapoptotic effect of EDC therapy in an in vitro culture system. Cellular viability was determined in cultured cardiomyocytes 12 hours after hypoxia. There was a significant increase in Annexin-V-positive cells after 12-hour hypoxia (Fig. 6) compared to the control group under normoxic conditions. However, the number of apoptotic cells significantly decreased in cardiomyocytes precocultured for 12 hours with EDCs, as indicated by a reduction the number of Annexin-V-positive cells.

DISCUSSION

It is known that, in the normal heart [18], the rate of cell death increases with age and is not balanced by a concomitant increase in new myocyte formation after middle age The excess cell death results in a net reduction in cardiomyocyte number [2,19] The remaining viable myocytes become hypertrophic to compensate for the reduced number of functional cardiomyocytes However, aged hypertrophic myocytes do not respond normally to growth stimuli and are prone to undergo cell death Apoptosis is much more frequent, particularly in hypertrophic myocytes expressing $p16^{INK4a}$ [19] Aged hearts also have impaired angiogeneic response as a result of a decreased ability to release platelet-derived growth factor [20] Our recent study demonstrated a significant decrease in myocardial blood perfusion and a reduction in the number of cardiomyocytes in aged hearts [2] The combination of age-dependent increase in myocyte death, coupled with reduction in vascular perfusion, further impairs functional adaptation in the senescent heart Apoptosis of cardiomyocytes has also been observed in cardiac tissue from patients with chronic cardiomyopathy later after MI [21] This observation leads to a concept that myocyte apoptosis contributes to the chronic progression of myocardial failure

In the past few years stem cells have been engrafted into a broad spectrum of tissues, including regenerating bone, neuron, dystrophic skeletal muscle, as well as cardiac tissue The biological principle is that stem cells contain a unique capacity of tissue-directed differentiation Our previous studies indicate that EDCs can differentiate into cardiac-like cells and improve cardiac function in postinfarcted adult rat hearts at 6 weeks [6] and 32 weeks [7] after cell transplantation More recently, we found that EDC transplantation restored the number of cardiomyocytes toward normal values [2] The present study demonstrated that transplantation of EDCs improved cardiac function and enhanced survival rate in the aging rats with MI during 6 months of observation The underlying mechanism might at least in part be attributed to the antiapoptotic effect of the implanted EDCs There is a growing body of evidence supporting cardiac regeneration and enhanced angiogenesis in infarcted hearts as a part of stem cell therapy However, the percentage of regenerated cardiac tissues is not sufficiently robust to significantly improve cardiac function after MI A linear regression analysis did not indicate a stronger correlation between a single variable (either myocyte number or blood flow) and ventricular function [2] Myogenesis, angiogenesis, as well as other factors (e g , the antiapoptotic role) may contribute synergistically to the functional benefit of stem cell transplantation In the present study we demonstrated that apoptosis of cardiomyocytes significantly increased 3 days after MI in the peri-ischemic myocardium EDC transplantation significantly reduced the number of apoptotic nuclei in the peri-infarcted

area compared to MI-Control group Furthermore, we found that there was a significant reduction in the number of apoptotic cardiomyocytes after 12 hours of hypoxia when they were cocultured with EDCs These results suggest that EDCs may rescue ischemia-induced apoptosis of cardiomyocytes and may partially explain an improvement in cardiac function at 6 months in the aging rats after MI and cell transplantation However, the detection of apoptotic nuclei by TUNEL staining has been challenged [22]. Electron microscopy showed that TUNEL-positive cardiomyocytes exhibited irreversible oncosis with ruptured plasma membranes [23] Thus, using electron microscopy in our future study to observe ultra-structural evidence of apoptosis should be important to address antiapoptotic effect of stem cell therapy

In an in vivo experiment in rats, DNA strand breaks were seen as early as 3 hours after MI [24] Apoptosis can be initiated by the mitochondria, which release cytochrome *c* into the cytosol in response to extrinsic stimuli, for example, oxidative insults The mitochondria-mediated pathway is modulated by the Bcl-2 family of proteins [25,26] At least 18 members of the Bcl-2 family have been identified, and these can be either pro-apoptotic (e g , Bax) or antiapoptotic (e g., Bcl-2). Apoptosis was accompanied by a decrease in Bcl-2 protein values and an increase in the protein levels of Bax, indicating a role of these proteins in the regulation of ischemia-induced apoptosis. In contrast, myocardial reperfusion injury was reduced in transgenic mice overexpressing Bcl-2 [27] The caspases are a group of cysteine proteases that play a crucial role in initiating and executing apoptosis A short prodomain-characterized effector, caspase 3, acts downstream in the common pathway to carry out the final biochemical change seen in apoptosis [28] Our present study shows that the protein levels of Bax and cleaved caspase 3 were significantly reduced in the aging rats that received EDC transplantation as compared to the MI-Control rats However, the protein levels of Bcl-2 increased significantly in infarcted rat hearts that received EDC transplantation at 3 days after treatment as compared to those in the hearts of the MI-Control rats. Our findings are consistent with those of many previous studies, which demonstrated that caspase 3, Bax, and Bcl-2 are involved in the process of apoptosis after cardiac ischemia

The underlying mechanism of antiapoptosis from engrafted EDCs remains unclear Cardiac-protecting factors released from implanted stem cells (e g , insulin-like growth factor [IGF]) appear to be the crucial to explain the antiapoptotic effect In neonatal rat cardiomyocytes, IGF prevents apoptosis after hypoxia, which also could be suppressed with genistein, a tyrosine kinase inhibitor [29] Subsequently, it has been found that IGF exerts its antiapoptotic effect through extracellular signal-regulated kinases (ERK1 and ERK2), resulting in activation of the transcription factor cyclic AMP response element binding protein, which induces Bcl-2 expression [30] A recently published article indicated that the release of basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF),

and stromal cell-derived factor-1 (SDF-1) from engrafted mesenchymal stem cells leads to efficient vascular regeneration and also attenuates the apoptotic pathway [31]. Another study reported that the release of paracrine growth factors from mesenchymal stem cells could promote neoangiogenesis [32]. Engrafted stem cells overexpressing Akt intended to increase stem cell viability that may have resulted from induced paracrine effects to perform antiapoptotic effects and salvage on adjacent ischemic myocardium [32]. There is growing evidence supporting the hypothesis that paracrine mechanisms mediated by factors released from stem cells play an essential role in the reparative process observed after the donor cells are injected into infarcted hearts, in addition to the direct regenerative potential of the stem cells. The low differentiation rate of transplanted stem cells to functional myocytes could not alone explain the structural and functional improvements reported. Based on the present study we believe that, in addition to cardiac regeneration by engrafted stem cells, myocardial salvage, derived from rescuing apoptotic cardiomyocytes through the paracrine mechanism, plays an important role in cardiac repair after stem cell transplantation.

Summary

Our data demonstrated that EDC transplantation improved cardiac function in aging rats at 6 months after MI. The antiapoptotic effect from EDC transplantation with either in vivo or ex vivo experiments may play an important role on the functional benefit. Further studies should be designed to clarify the specific cardioprotecting factors released from engrafted stem cells and determine their potential for therapeutic application in preventing cardiac apoptosis during myocardial ischemia or infarction. Thereafter, instead of transplanting stem cells, it may be possible to administer specific proteins made by stem cells for cardiac repair in the aged population.

ACKNOWLEDGEMENTS

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CHAPTER 6

Angiotensin II promotes fusion of embryonic stem cells with adult cardiomyocytes

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ABSTRACT

The role of T-type Ca^{2+} channels in fusion of embryonic stem cells with adult cardiomyocytes has not yet been clarified. Here, we studied the effects of angiotensin II-induced T-type Ca^{2+} channel expression on fusion of embryonic stem (ES) cells with cardiomyocytes. Green fluorescent-labeled mouse ES cells were co-cultured with red fluorescent-labeled isolated adult rat cardiomyocytes ES cells to determine cell fusion. We used Cre/lox recombination to confirm cardiomyocyte fusion with ES cells, and administered angiotensin II (Ang II) to cardiomyocytes to induce expression of T-type Ca^{2+} channels. T-type Ca^{2+} channel current was recorded using the whole-cell patch clamp technique. Then changes in intracellular Ca^{2+} $[Ca^{2+}]_i$ were measured using fura-2 fluorescence. Double fluorescent (red/green) adult cardiomyocytes were detected, indicating fusion with ES cells. Cell fusion was confirmed using Cre/lox recombination. Ang II-induced expression of T-type Ca^{2+} channels significantly increased cell fusion. Expression of T-type Ca^{2+} channels was associated with a significant increase in $[Ca^{2+}]_i$. Coadministration of the T-type Ca^{2+} channel blocker amiloride inhibited Ang II-induced expression of T-type Ca^{2+} channels, the increase in $[Ca^{2-}]_i$ and cell fusion. Our results suggest that Ang II-induced expression of T-type Ca^{2+} channels promotes fusion of ES cells with adult cardiomyocytes.

INTRODUCTION

Stem cell transplantation has been shown to improve function of injured myocardium in animals and in man [1,2]. The achieved functional improvement appears to be disproportionate to the corresponding number of stem cell-derived cardiomyocytes, however, indicating that multiple mechanisms contribute to the improvement following stem cell transplantation. [3-7].

Recent studies suggest that activation of various signaling pathways, including intracellular Ca^{2+} exchange, are involved in cell fusion [8-11]. Fusion of skeletal muscle myoblasts, one of the first donor cell types used in an animal model [12], are known to be a Ca^{2+}-dependent process [13]. Bijlenga et al. have shown that Ca^{2+} entry through T-type Ca^{2+} channels is required for skeletal muscle myoblast fusion, and that T-type Ca^{2+} channels are expressed just before fusion [14]. Hyperpolarization of the membrane potential produces a T-type Ca^{2+} 'window' current that promotes Ca^{2+} influx and triggers fusion [15,16]. Inhibition of T-type Ca^{2+} channels by amiloride prevents an increase in intracellular Ca^{2+} and blocks myoblast fusion [14,17].

T-type Ca^{2+} channels are expressed in embryonic and neonatal hearts in both atrial and ventricular myocytes, but down-regulated in adult ventricular myocytes [18,19] T-type Ca^{2+} channels, however, are re-expressed in hypertrophied adult ventricular myocytes [20-23] Expression of T-type Ca^{2+} channels has previously been shown in embryonic stem cell-derived cardiomyocytes indicating that T-type Ca^{2+} channels play an important role in cardiomyocyte differentiation [24,19] T-type Ca^{2+} channel expression has also been shown to increase after treatment with Ang II [25-27]

Taken together, T-type Ca^{2+} channels appear to promote fusion of cardiomyocytes with stem cells, but this has not yet been definitively determined Moreover, identification of agents that can promote fusion could significantly advance ES cells as a promising therapy

Here, therefore, we studied fusion of ES cells with isolated adult rat cardiomyocytes, and whether Ang II-induced expression of T-type Ca^{2+} channels enhanced cell fusion

MATERIALS AND METHODS

Culture of ES cells and adult cardiomyocytes

The mouse ES cell line, ES-D3, was obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained as previously described [2] Briefly, ES-D3 cells were cultured in Dulbecco modified Eagle medium on mitotically inactive mouse embryonic fibroblast feeder cells (ATCC, Manassas, VA) The medium was supplemented with 10% fetal bovine serum (ATCC, Manassas, VA), 0 1 mM 2-mercaptoethanol (Invitrogen, Carlsbad, CA), and 1000 units/ml leukemia inhibitory factor (Sigma-Aldrich, Saint Louis, MO) The 6-well plates were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 Growth medium was changed twice weekly and cells were subcultured weekly by dissociation with trypsin for 3 min

Adult cardiomyocytes were harvested from 8-week-old Wistar rats as previously described [28] Animal studies were performed with the approval of the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center

Cell fusion determined by fluorescence microscopy

ES cells labeled with green fluorescent 5,6-carboxyfluorescein diacetate succinimidyl ester (CFDA SE) were co-cultured with adult cardiomyocytes labeled with red fluorescent 1,1'-dioctadecyl-3,3,3',3'- tetramethylindocarbocyanine (DiI) (Molecular Probes, Inc , Eugene, OR) for 3 days at a 1 1 ratio (10^5 cells) Images were obtained using an inverted fluorescence microscope at a magnification of 20x (TE2000-U, Nikon, Tokyo, Japan) Images were

analyzed using IPLab software (Scanalytics, Inc., Fairfax, VA). Double fluorescent cells indicating fusion of ES cells with adult cardiomyocytes were counted in each section by an inverted fluorescence microscope at a magnification of 10x (TE2000-U; Nikon, Tokyo, Japan) equipped with a Photometrics Cool Snap HQ charge-coupled device camera (Roper Scientific, Trenton, NJ) (7 cultures, ~10,000 cells per condition). Results are presented as the percentage of double fluorescent cells per condition.

Cell fusion determined by Cre/lox recombination

We used the Cre/lox recombination system (Microbix Biosystems, Inc., Toronto, Canada) to confirm fusion of ES cells with adult cardiomyocytes. ES cells were infected with LacZ adenovirus (300 particles/cell) and adult cardiomyocytes were infected with the Cre-adenovirus (300 particles/cell) (Microbix Biosystems Inc, Toronto, Canada). The infected cells were washed three times with PBS and then cocultured for 3 days. The cells were fixed with 3% paraformaldehyde and stained with 5-bromo-4-chloro-3-indolyl-D-galactoside (X-gal). Blue-stained cells indicating cell fusion were visualized at a magnification of 20x with a Nikon E800 (Nikon, Tokyo, Japan) coupled to a Spot-CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI) using Image-Pro Plus analysis software (Media Cybernetics, Silver Spring, MD) (5 cultures).

Administration of Ang II, amiloride and verapamil

Adult cardiomyocytes were administered Ang II (0.1 μmol/l) (Sigma Chemicals, St. Louis, MO) 48 hours before co-culture with ES cells. A separate group of Ang II-treated adult cardiomyocytes were administered the T-type Ca^{2+} channel blocker, amiloride (3 μmol/l), or the L-type Ca^{2+} channel blocker, verapamil (10 μmol/l), daily during 3 days of co-culture to determine their effects on T-type Ca^{2+} channel expression (7 cultures, ~10,000 cells per condition).

Measurement of cardiomyocyte size

Control and Ang II-treated adult cardiomyocytes were imaged with an inverted fluorescence microscope at a magnification of 10x (Nikon, Tokyo, Japan) and analyzed by IPLab software (Scanalytics Inc., Fairfax, VA). Cardiomyocyte size was determined by measuring cell length and width (~70 cardiomyocytes from 7 cell cultures).

Electrophysiological recordings

Adult cardiomyocytes were prepared for electrophysiological recordings as previously described [28]. Whole-cell recordings were performed using the standard patch clamp technique at room temperature (20° C) [29,30]. The external recording solution contained (in mM): $CaCl_2$, 1.8; tetraethylammonium chloride (TEA-Cl), 130; KCl, 5; Hepes, 5; $MgCl_2$, 2; glucose, 8; tetrodotoxin, 0.01; 4-aminopyridine, 2; nifedipine, 0.01 (pH 7.4). The pipette solution contained (in mM): KCl, 115; TEA-Cl, 30; $MgCl_2$, 2; Hepes, 10; glucose, 5; 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, 1 (pH 7.3). Ca^{2+} current was elicited by step depolarization from holding potentials of -80 mV and -50 mV to test potentials of -40 mV, -20V and 10 mV using an Axoclamp 2B amplifier (Axon Instruments, Union City, CA). T-type Ca^{2+} current was measured by taking the difference between currents recorded from holding potentials of -80 mV and - 50 mV. T-type Ca^{2+} current was recorded in controls, Ang II-treated and Ang II plus amiloride-treated adult cardiomyocytes (12 cells from 4 separate cultures per condition).

Calcium measurement

We used the fura-2 fluorescence method to determine changes in intracellular calcium [Ca2+]i concentration [31]. Adult cardiomyocytes were loaded with 5μmol/l fura-2 AM (Molecular Probes, Eugene, OR) for 40 min at room temperature (20°C). Cells were washed free of extracellular fura-2 AM and suspended in Medium 199 (pH 7.4). Cells were cultured with Ang II (0.1 μmol/l) or Ang II plus amiloride (3 μmol/l) for 48 hours prior to fura-2 AM loading (4 cells from 5 separate cultures per condition). Fura-2 fluorescence was excited alternately at 340 and 380 nm wavelengths using a filter wheel and a 75 W xenon lamp. A shutter prevented illumination of the cells to reduce photo-bleaching, except during data acquisition. Fura-2 fluorescent emission was acquired through a 510 nm dichroic mirror and 520 nm long pass filter set (Chroma Technology, Brattleboro, VT). Fluorescent images were captured at 200 ms intervals and background was subtracted from each image. The images were viewed with a fluorescence microscope at a magnification of 20x (TE2000-U; Nikon, Tokyo, Japan). Changes in $[Ca2+]_i$ were determined using the ratio of fluorescence of the two excitation wavelengths, 340 and 380 nm (~20 cells per condition).

Statistical analysis

Results are presented as mean±S.E.M. Comparison between groups was performed using one-way ANOVA. When significant group differences were observed, Student's two-tailed *t*-test for unpaired observations was performed. Differences were considered significant with $P<0.05$.

RESULTS

Cell fusion

We co-cultured ES cells labeled with CFDA SE (green fluorescence) with adult cardiomyocytes labeled with Dil (red fluorescence) for 3 days (7 cultures, ~10,000 cells per condition). We found 0.07±0.02% double fluorescent cells (red/green) indicating fusion of ES cells with adult cardiomyocytes (Fig. 1). We used Cre/lox recombination to confirm fusion of ES cells with cardiomyocytes. We cocultured LacZ adenovirus-infected ES cells with Cre-adenovirus-infected adult cardiomyocytes and stained the cocultured cells with X-gal (Fig. 1).

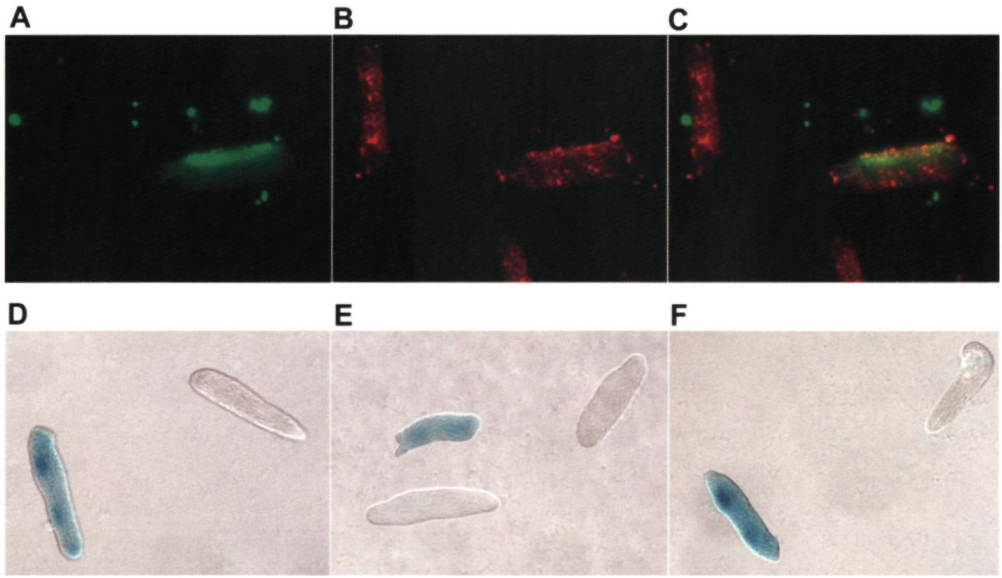

Figure 1. Fusion of ES cells with adult cardiomyocytes. We co-cultured ES cells labeled with CFDA SE (green fluorescence) with adult cardiomyocytes labeled with Dil (red fluorescence) for 3 days (7 cultures, ~10,000 cells per condition). We found double fluorescent cells (red/green) indicating fusion of ES cells with adult cardiomyocytes. (**A)** Green fluorescent-labeled ES cells plus cardiomyocyte (x20). (**B)** Red-fluorescent-labeled cardiomyocytes (x20). (**C)** Double fluorescent-labeled cardiomyocyte, green fluorescent-labeled ES cells and red-fluorescent-labeled cardiomyocytes (x20).
We cocultured LacZ adenovirus-infected ES cells with Cre adenovirus-infected adult cardiomyocytes. *T*he cultured cells were stained with X gal. (**D–F)** X-gal-positive cells (blue) indicating fusion of ES cells with cardiomyocytes (x20).

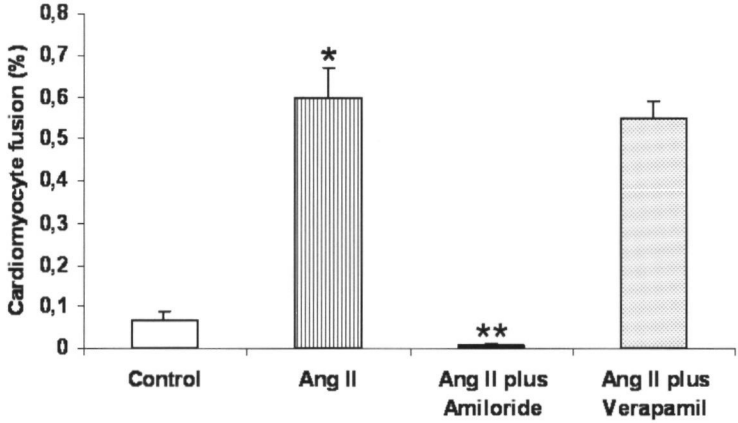

Figure 2. Fusion of ES cells with Ang II-treated adult cardiomyocytes. Ang II significantly increased fusion of ES cells with Ang II-treated cardiomyocytes compared to controls. Co-administration of the T-type calcium channel blocker amiloride abolished the increase in Ang II-induced cell fusion whereas co-administration of the L-type calcium channel blocker verapamil did not inhibit cell fusion. (Mean±S.E.M., *P<0.05 vs. control, **P<0.05 vs. Ang II plus amiloride, ~10000 cells per condition).

Ang II enhanced cell fusion

We administered Ang II to adult cardiomyocytes to induce hypertrophy and re-expression of T-type Ca^{2+} channels. Administration of Ang II significantly increased cardiomyocyte length to 105±2 μm compared to 96±2 μm in controls, and cardiomyocyte width to 31±1 μm compared to 21±1 μm in controls (P<0.05, 7 cultures, 70 cells per condition) (Fig. 2).

It has been shown that Ang II increased expression of T-type Ca^{2+} channels [27]. We cocultured CFDA SE-labeled ES cells with Dil-labeled Ang II-treated adult cardiomyocytes to detect fusion of ES cells with cardiomyocytes. We found that administration of Ang II significantly increased fusion of ES cells with cardiomyocytes to 0.56±0.07 % compared to 0.07±0.02% in Ang II-untreated controls (P<0.05) (Fig. 2). Coadministration of the T-type Ca^{2+} channel blocker amiloride abolished the increase in Ang II-induced cell fusion whereas co-administration of the L-type Ca^{2+} channel blocker verapamil did not inhibit fusion (P<0.05, ~10000 cells per condition) (Fig. 2). These findings suggest that the increase in fusion of ES cells with Ang II-treated cardiomyocytes may be mediated by re-expression of T-type Ca^{2+} channels.

Ang II induced T-type Ca^{2+} current expression

Previous studies have shown that cardiac T-type Ca^{2+} current is re-expressed in hypertrophied ventricular cells [20-22]. Lu et al. reported that Ang II enhanced T-type Ca^{2+} current [26]. We recorded T-type Ca^{2+} current in Ang II-treated adult cardiomyocytes using the whole-cell patch clamp technique. The membrane potential was depolarized to -0.59±0.05 mV in Ang II-treated cardiomyocytes compared to -0.75±0.03 mV in controls ($P<0.05$). We found expression of T-type Ca^{2+} current in the majority of Ang II-treated cardiomyocytes. Coadministration of the T-type Ca^{2+} channel blocker, amiloride, abolished T-type Ca^{2+} current expression (12 cells from 4 different cultures per condition (Fig. 3).

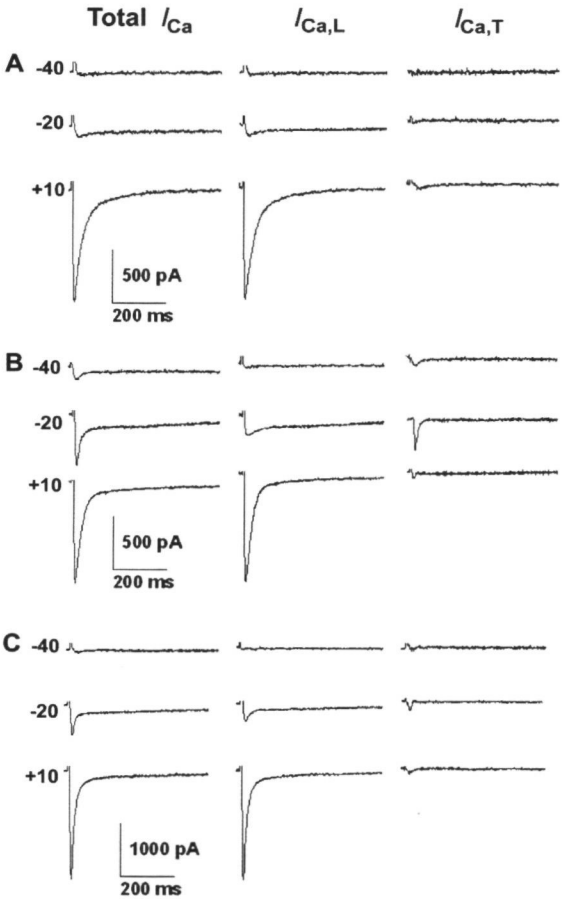

Figure 3. T-type Ca^{2+} current ($I_{Ca,T}$) in adult cardiomyocytes.
Total I_{Ca} and $I_{Ca,L}$ were measured by using holding potentials of -100 mV and –50 mV, respectively. $I_{Ca,T}$ was obtained by subtraction of $I_{Ca,L}$ from total I_{Ca}.
A. There was no expression of $I_{Ca,T}$ in control cardiomyocytes. **B.** Ang II administration induced expression of $I_{Ca,T}$. **C.** Coadministration of the T-type calcium channel blocker amiloride inhibited expression of $I_{Ca,T}$.
(Total I_{Ca}, whole-cell calcium current; $I_{Ca,L}$, L-type Ca^{2+} current; $I_{Ca,T}$, T-type T-type Ca^{2+} current; 12 cells from 4 different cultures per condition).

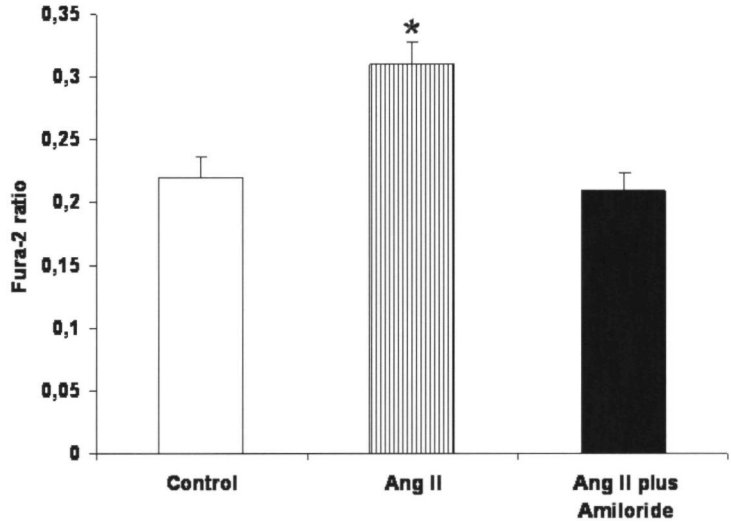

Figure 4. Changes in intracellular calcium $[Ca^{2+}]_i$ concentration. Ang II significantly increased the ratio of fura-2 fluorescence in adult cardiomyocytes compared to controls indicating a significant increase in $[Ca^{2+}]$ via the T-type calcium channel. Co-administration of the T-type calcium channel blocker amiloride abolished the increase in Ang II-induced $[Ca^{2+}]_i$. (Mean±S.E.M., *P<0.05 vs. control, ~20 cells per condition).

Ang II-induced expression of T-type Ca^{2+} current was associated with a significant increase in $[Ca^{2+}]_i$. The ratio of fura-2 fluorescence increased from 0.22±0.02 in controls to 0.31±0.02 in Ang II-treated cells. Amiloride decreased the ratio of fura-2 fluorescence to 0.21±0.02 (P<0.05, 4 cells from 5 separate cultures per condition) (Fig. 4).

DISCUSSION

ES cells hold great promise as a therapeutic approach for cardiovascular disease, but their fusion with cardiomyocytes is essential. Techniques to enhance the phenomenon are necessary to advance ES cells as being a dependable therapeutic option. Our findings suggest that Ang II-induced T-type Ca^{2+} channel expression in adult cardiomyocytes enhanced fusion with ES cells. Expression of T-type Ca^{2+} channels was associated with an increase in $[Ca^{2+}]_i$ The T-type Ca^{2+} channel blocker amiloride abolished the increase in $[Ca^{2+}]_i$ and cell fusion.

It has been previously shown that transplanted mouse ES cells differentiated into cardiomyocytes and improved cardiac function [2,32,33]. Possible mechanisms of stem cells to transform into cardiomyocytes include: stem cells transdifferentiate into cardiac muscle cells [34-36], stem cells mutate to cardiomyocytes [37,38], or stem cells fuse with cardiomyocytes [34,36,37,39]. ES cells that fused with fetal cardiomyocytes exhibited phenotype and proliferation characteristics of cardiomyocytes [40]

Zhang et al found that ~1% of human peripheral blood CD34-positive cells fused with cardiomyocytes in SCID mice [36]. OH et al. have reported that ~0.8% of adult cardiac progenitor cells (α-MHC-Cre Sca-1$^+$ cells) grafted into the myocardium of Rosa26 Cre reporter mice [6]. Others have shown that grafting of loxed-lacZ C2C12 skeletal myoblasts into normal hearts of α-MHC-Cre$^+$ mice resulted in ~0.01% of fused skeletal myoblasts with cardiac cells [7].

These findings are consistent with our observations. We found that ~0.07% of ES cells fused with adult cardiomyocytes (Fig. 1). Our findings further demonstrate that pretreatment with Ang II significantly increased fusion of ES cells with cardiomyocytes (Fig. 2).

T-type Ca^{2+} channels are expressed in neonatal ventricular cardiomyocytes but are down-regulated in adult cardiomyocytes [18,19]. However, models of pressure overload-induced hypertrophy and postinfarction-remodelled hypertrophied left ventricles have demonstrated that T-type Ca^{2+} channel gene and current are re-expressed [21-23]. Izumi et al. observed that T-type Ca^{2+} current reappeared in failing ventricular cardiomyocytes of Dahl salt-sensitive rats with hypertension [25]. Ferron et al. have shown that expression of T-type Ca^{2+} current was mediated by Ang II signaling pathways in hypertrophied cardiomyocytes of aortic-banded rats [20]. The authors further reported that Ang II administration (0.1 μmol/l) increased the expression of T-type Ca^{2+} current in cultured newborn rat cardiomyocytes. Others have shown that Ang II increased expression of T-type Ca^{2+} current in cultured cells [26,27,41]. Our findings are consistent with these reports and suggest that Ang II administration (0.1 μmol/l) resulted in expression of T-type Ca^{2+} current in isolated adult rat cardiomyocytes (Fig. 3). We found that T-type Ca^{2+} current expression was present in the majority of Ang II-treated adult cardiomyocytes.

It has been previously shown that myoblast fusion and subsequent myotube formation is a Ca^{2+}-dependent process, as it is inhibited by low extracellular or intracellular Ca^{2+} concentrations [42]. Further studies have demonstrated that in skeletal muscle myogenesis fusion of myoblasts and myotube formation is a process that requires $[Ca^{2+}]$, influx through T-type Ca^{2+} channels [14,43,44]. Inhibition of T-type Ca^{2+}channel activity by amiloride prevented both the increase in $[Ca^{2+}]$, in fusion-competent myoblasts and the process of fusion [14, 17]. Our findings are consistent with these data and suggest that expression of T-type Ca^{2+} channels increased fusion of cardiomyocytes with ES cells. Our data further show that

the T-type Ca^{2+} channel blocker amiloride inhibited cell fusion, whereas the L-type Ca^{2+} channel blocker verapamil had no effects on fusion (Fig. 2).

Previous studies have shown that hyperpolarization of the resting membrane potential is required for Ca^{2+} influx and initiating myoblast fusion by a window current through T-type $\alpha 1H$ Ca^{2+} channels [14,45,46]. Arnaudeau et al. have shown in rat portal vein myocytes that depolarization to -10 mV from a holding potential of −50 mV produced Ca^{2+} currents of small amplitude (20–30 pA) which produced local $[Ca^{2+}]_i$ transients similar in amplitude and time course to spontaneous Ca^{2+} sparks. The authors further showed that Ang II administration resulted in activation of several Ca^{2+} sparks leading to a sustained increase in $[Ca^{2+}]_i$ [47]. Guibert et al. have demonstrated that Ang II-induced oscillations in $[Ca^{2+}]_i$ increased the resting $[Ca^{2+}]_i$ concentration in rat pulmonary artery myocytes by stimulation of AT1 receptors [29]. Our findings are consistent with these observations. We found that Ang II treatment depolarized the membrane potential to -0.59 mV compared to -0.75 mV in Ang II-untreated cardiomyocytes, which was associated with an ~ 45 % increase in resting $[Ca^{2+}]_i$.
In conclusion, our results show that Ang II-induced expression of T-type Ca^{2+} channels increased fusion of ES cells with adult cardiomyocyte.

Our study further demonstrates that increased Ca^{2+} influx through the T-type Ca^{2+} channel may play an important role in promoting cell fusion. The present study may provide a basis to explore the potential for T-type Ca^{2+} channels to enhance cell fusion to regenerate damaged myocardium.

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CHAPTER 7

Stem cell therapy in the aging hearts of Fisher 344 rats: Synergistic effects on myogenesis and angiogenesis

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ABSTRACT

Advanced age is a major risk factor for ventricular dysfunction and reduction of cardiac reserve. Finding novel approaches to prevent and attenuate heart dysfunction associated with advanced age is a major therapeutic challenge. The present study was designed to test whether engrafted embryonic stem cells could improve myocardial function in aging hearts. Cultured mouse embryonic stem cells used for cell therapy were transfected with green fluorescent protein. Aging rats in the cell-treated group received intramyocardial injection of embryonic stem cells. Hemodynamic measurement, myocyte counting, and evaluation of blood flow were performed 6 weeks after cell transplantation. Embryonic stem cell therapy partially improved cardiac reserve, as reflected by the in vivo response to isoproterenol (INN: isoprenaline) stimulation in aging hearts 6 weeks after cell implantation. The functional benefits from engrafted embryonic stem cells were associated with increased myocyte numbers and enhanced left ventricular blood perfusion in the aging heart. The characteristic phenotype of engrafted embryonic stem cells was identified in the transplanted heart on the basis of green fluorescent protein-positive spots that were further demonstrated to differentiate into cardiac tissue with positive staining for cardiac α-myosin heavy chain. Regenerating cardiomyocytes and increasing regional blood perfusion in the aging heart after embryonic stem cell transplantation synergistically resulted in improvement of cardiac function. Embryonic stem cell transplantation might hold significant clinical potential in attenuating the progressive decrease of cardiac function associated with advanced aging.

INTRODUCTION

Epidemiologic studies have shown that congestive heart failure is the major cause of most hospitalizations of persons older than 65 years [1,2]. Heart transplantation is an acceptable alternative for treating end-stage heart failure in addition to drug treatment; however, only a small number of patients can receive heart transplantation because of the shortage of donors. Therefore finding novel and effective approaches to prevent and reduce the pathogenesis of heart dysfunction associated with advancing age is a major therapeutic challenge.

It has been demonstrated through the last decade that the aging process of the heart in animals [3,4] and human subjects [5] is characterized by a significant loss of cardiac myocytes, especially in the left ventricle. A recent study [6] documented that aging hearts have impaired angiogenic function as a result of decreased production of a platelet-derived

growth factor. Impaired angiogenesis in aging hearts might facilitate the myocyte loss and subsequently reduce heart function. Recently, therapeutic approaches aimed at promoting angiogenesis and regenerating new cardiac myocytes have been undergoing intensive investigation. Engrafted cells have been shown to survive, proliferate, and form gap junctions with the host myocardium [7,8] and partially restore the impaired cardiac function in either cryoinjured [9] or infarcted [10] hearts. Our recent studies demonstrated that embryonic stem (ES) cells can differentiate into cardiac-like cells and improve cardiac function in postinfarcted rat hearts at 6 weeks [11] and 32 weeks [12] after cell implantation. The present study was designed to determine whether ES cells after intramyocardial implantation could regenerate new cardiac myocytes and enhance cardiac function in aging hearts.

MATERIALS AND METHODS

ES cell preparation and transplantation

The mouse ES cell line ES-D3 was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured with a previously described method [11,12]. Before transplantation, cells dissected from beating clusters were transfected with green fluorescent protein (GFP), a marker for the identification of engrafted cells. Two days after GFP transfection, cultured ES cells were trypsinized and resuspended in Joklik modified medium (Sigma, St. Louis, MO) with a density of $2x10^7$ cells/mL for cell transplantation.

Experiments were performed in 46 senescent male Fisher 344 rats aged 24 months (obtained from the National Institute on Aging) and 24 adult male Fisher 344 rats aged 3 months (Charles River, Wilmington, MA). The investigation conformed to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (National Institutes of Health publication no. 85-23, revised 1996), and the protocol was approved by our institutional animal care committee. Animals were intubated after achievement of anesthesia with pentobarbital (60 mg/kg administered intraperitoneally) and ventilated with room air by using a small animal ventilator (Harvard Apparatus, South Natick, MA). Intramyocardial injection of the ES cell suspension (50 μl, approximately 10^6 cells) was performed in 3 different sites (1 in the apex and 2 in the middle of the left ventricle) with a tuberculin syringe. The adult and aging control rats received the exact volume of the cell-free medium. Two additional aging rats receiving stem cell injection were used for monitoring cardiac arrhythmia. The electrocardiographic (ECG) transmitter (Data Sciences International Inc., St. Paul, MN) was implanted in the rat abdominal cavity with a previously described method [13] and the signal was collected by a receiver (Data Sciences International Inc.).

Functional measurement and β-adrenergic responsiveness in vivo

Rats (10 for each group) were anesthetized with pentobarbital (60 mg/kg administered intraperitoneally) at 6 weeks after cell transplantation. Hemodynamic measurements in vivo were performed with a modified method, as described previously [11,12]. After hemodynamic measurements at baseline, serially increasing intravenous bolus injections of isoproterenol were given in doses of 0.1, 0.3, 1.0, 3.0 and 6.0 μg/kg through the femoral vein to determine the inotropic responsiveness to β-adrenergic stimulation. The doses of isoproterenol used in the present study were chosen on the basis of a previous report [14]. Hemodynamic responses to β-adrenergic stimulation were collected at 8- to 10-minute intervals to obtain a dose-response curve.

Regional blood flow measurement and numeric density of arterioles

Stable, nonradioactive isotope-labeled microspheres (15 μm; BioPAL Inc., Worcester, MA) were used to determine the regional blood flow in anesthetized rats 20 minutes after measurement of hemodynamics and β-adrenergic stimulation. The method was modified from a previous publication [15]. In brief, a set of microspheres (1.25×10^6 in 0.5 ml) was diluted in 0.5 ml of sanSaline saline (BioPAL Inc.) and injected into the left atrium over 10 seconds during cardiac surgery. Reference blood samples were withdrawn by using a syringe at a constant rate of 2-minute intervals through the femoral artery, resulting in a 2-ml sample used to calculate absolute myocardial blood flow. The rat heart was then harvested during deep anesthesia. The heart was weighed and normalized by body weight. The left ventricle was surgically isolated and cut into transmural slices. Two segments from the middle of the left ventricle and one from the apex were used for blood flow measurement. The average myocardial sample weighed approximately 0.15 g. A piece of the right ventricle was isolated for tissue control reference. Finally, the tissue and blood samples were shipped to BioPAL Inc. for measurement of active isotope microspheres and determination of myocardial regional blood flow.

The numeric density of arterioles (diameter >20 μm) was counted in each area observed (5 for each group) on hematoxylin and eosin-stained slides by using a light microscope at 400x magnification. Five high-power fields in the transplanted area were randomly selected; the number of arterioles in each section was averaged and expressed as the number of arterioles per unit area (in square millimeters).

Isolated myocytes and myocyte number counting

In another set of experiments (7 adult control, 6 aging control, and 6 aging rats with cell injection), rats were anesthetized with pentobarbital 6 weeks after cell transplantation. The

procedure of myocyte isolation was modified from a method described previously [16] Isolated myocytes were counted with a hemocytometer using a microscope Hemodynamics were measured as described above before dissecting cardiomyocytes for linear regression study

Identification of transplanted cells, histologic analysis, and Western blotting

Five additional rats for each group were used for histologic study The hearts were harvested after achievement of anesthesia and subsequently dissected into four transverse sections from apex to base Five-micrometer transverse slices from each section were prepared for hematoxylin and eosin staining The survival of engrafted cells was identified on the basis of GFP-positive clusters from the aging hearts with stem cell injection Transformation of cardiac-like cells from engrafted ES cells was verified by means of antibody immunostaining for cardiac-specific α-myosin heavy chain (α-MHC) Briefly, prepared sections were incubated with a mouse anti-α-MHC monoclonal antibody (Berkeley Antibody Co , Richmond, CA) for 60 minutes at room temperature After washing with PBS, sections were incubated with rabbit anti-goat-conjugated rhodamine IgG Immunostaining was performed on serial sections of the rat heart Furthermore, the protein levels of sarcoplasmic reticulum Ca^2 ATPase (SERCA2) were measured in additional left ventricles (5 for each group) with a previously described method [17] to evaluate the activity of cardiac protein

Table 1. General characteristics of adult rats and aging rats with or without stem cell therapy

	Adult	Aging	Aging + ES cells
BW (g)	352±10	413±15	424±22
HW (g)	0 9±0 1	1 3±0 2	1 2±0 3
HW/BW100	0 2±0 02	0 3±0 03	0 3±0 04
HR (beats/min)	360±28	372±29	375±26
LVSP (mmHg)	126±15	108±11	112±10
LVEDP (mmHg)	5 8±0 7	11 2±1 6†	8 4±1 1†
$+dP/dt_{max}$ (mmHg)	19,296±954	9218±806	9626±915
$-dP/dt_{max}$ (mmHg)	9329±870	8017±475	8798±520†

Values are presented as means±SD Measurements were conducted at 6 weeks after ES cell or medium injection in 10 rats for each group BW, Body weight, HW, heart weight, HW/BW, ratio of heart weight/body weight, HR, heart rate, LVSP, left ventricular systolic pressure, LVEDP, left ventricular end-diastolic pressure, $+dP/dt_{max}$, rate of peak left ventricular systolic pressure increase, $-dP/dt_{max}$, rate of peak left ventricular systolic pressure decrease $P<0$ 05 and $^{\dagger}P<0$ 01 versus adult group,‡ $P<0$ 05, aging + ES cell group versus aging group

Statistical analysis

All data are presented as means±SD. Differences between groups were evaluated by means of 1-way analysis of variance. Means shown to be different between individual groups were compared with paired or unpaired Student's *t*-tests. The results of isoproterenol stimulation were analyzed by using 2-way analysis of variance. Linear regression modeling was used to compare parameters of the left ventricular systolic pressure (LVSP) and regional blood flow and parameters of the LVSP and the number of single isolated myocytes.

RESULTS

General characteristics and ventricular function after ES cell therapy

Three aging control rats with significant weight loss in the follow-up period before death were excluded. No tumors were seen during necropsy at the end of the study in any animals. During the follow-up period, we did not find significant cardiac arrhythmias with the telemetric ECG monitoring (data not shown). Our data indicated decrease of cardiac function in the aging hearts (Table 1) reflected by a mild reduction of the LVSP, the peak rate of the LVSP increase ($+dP/dt_{max}$), and the peak rate of the LVSP decrease ($-dP/dt_{max}$). However, the left ventricular end-diastolic pressure was significantly increased in aging rats compared with that seen in adult rats. Six weeks after intramyocardial injection of ES cells, the ventricular function was improved, accompanied by significantly reduced left ventricular end-diastolic pressure (Table 1). Isoproterenol stimulation induced a dose-dependent increase in the LVSP (Fig. 1) in adult control rats, whereas this positive inotropic effect was markedly blunted the aging heart. ES cell transplantation partly restored the inotropic response to isoproterenol in aging rats 6 weeks after cell injection (Fig. 1).

Left ventricular blood flow and myocyte regeneration after ES cell therapy

The regional blood flow measured by using stable, nonradioactive isotope microspheres was significantly decreased in the aging heart without cell treatment (Fig. 2A) compared with that seen in the adult heart. Cell transplantation significantly increased the left ventricular myocardial blood perfusion compared with that seen in the aging heart without cell injection. Regression analysis revealed no significant linear relationship (P=0.51) between the LVSP and the left ventricular blood flow (Fig. 2B). Additionally, the number of arterioles increased significantly in the aging heart after ES cell transplantation compared with that seen in the aging heart without cell therapy (7.0±1.3 arterioles/mm^2 in the aging plus ES cell group vs 5.2±0.7 arterioles/mm^2 in the aging group, P<0.05), although the arteriolar density was still

lower than that from the adult heart (7.0±1.3 arterioles/mm^2 in the aging plus ES cell group vs 10.8±1.7 arterioles/mm^2 in the adult group, $P<0.05$).

The average yields of myocytes in aging left ventricles without ES cell treatment were significantly lower than the number of myocytes yielded from adult rat left ventricles (Fig. 3A). Cell transplantation partially restored the number of myocytes in the aging rat left ventricles compared with that from aging rat left ventricles without cell therapy. Regression analysis showed LVSP and myocyte number to have a slightly positive association, but this did not reach statistical significance (Fig. 3B).

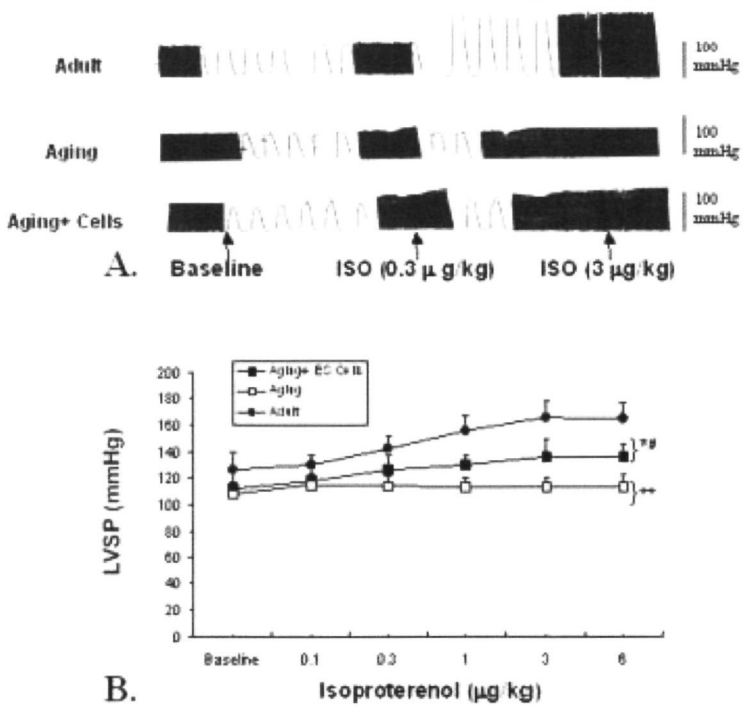

Figure 1. Inotropic response of the LVSP to isoproterenol (ISO) stimulation during hemodynamic measurements in vivo. Original representative recordings (**A**) show the response to isoproterenol stimulation was blunted in the aging heart without cell treatment. ES cell transplantation partially restored the inotropic response to β-adrenergic stimulation 6 weeks after cell injection. Summarized data are shown in panel **B**. *$P<0.05$ and **$P<0.01$ versus adult group; $^{\#}P<0.05$, aging + ES cell group versus aging group.

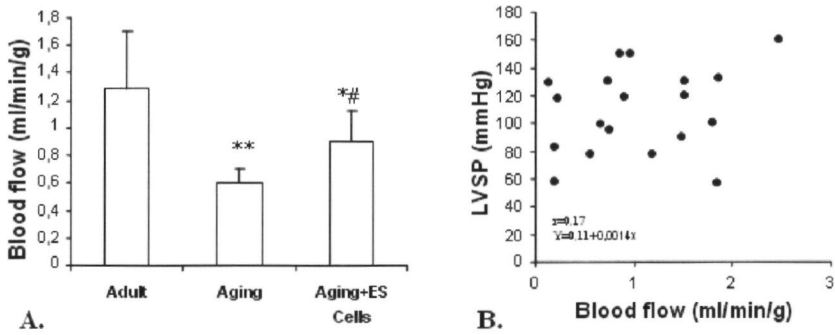

Figure 2. The results of the left ventricular blood flow measurements with isotope microspheres are shown (**A**). ES cell transplantation significantly restored the regional blood flow compared with the aging heart without cell treatment. A linear regression study (**B**) demonstrated a weak association between the LVSP and the regional blood flow. **P*<0.05 and ***P*<0.01 versus adult group; #*P*<0.05, aging+ES cell group versus aging group.

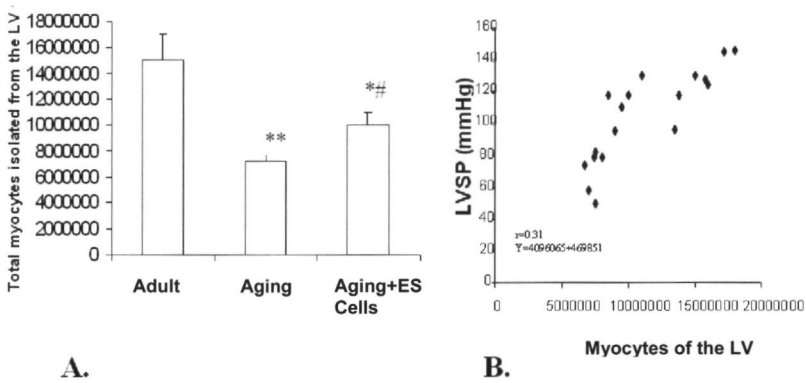

Figure 3. Myocyte counts from the adult and the aging heart with or without cell transplantation (**A**). The aging heart without cell therapy showed significant loss of cardiac myocytes. ES cell transplantation partially restored the myocyte number toward normal values. A linear regression study (**B**) demonstrated a moderate association between the LVSP and the number of myocytes. LV, left ventricle. **P*<0.05 and ***P*<0.01 versus adult group; #*P*<0.05, aging+ES cell group versus aging group.

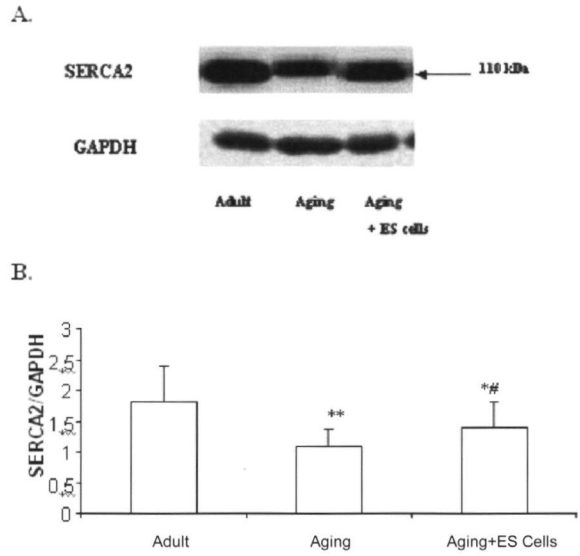

Figure 4. Protein expressions of SERCA2 (5 for each group) in left ventricles from adult rats, aging rats, and aging rats with cell transplantation determined by means of the Western blot technique. **(A)** Western blot; **(B)** densitometric analysis of SERCA2/reduced glyceraldehyde-phosphate dehydrogenase (GAPDH). **P*<0.05 and ***P*<0.01 versus adult.

Protein levels of SERCA2 after ES cell therapy, identification of implanted ES cells, and histologic study

The protein levels of SERCA2 were analyzed as a ratio compared with reduced glyceraldehyde-phosphate dehydrogenase (GAPDH). As shown in Figure 4, there was a moderate reduction of SERCA2 protein levels in aging left ventricles compared with that from adult left ventricles. ES cell treatment resulted in partially restored protein levels of SERCA2 in left ventricles from aging rats at 6 weeks after cell injection. Figure 5A and B, shows engrafted ES cells distributed within the host myocardium. Those regenerated tissues were not found in either adult myocardium or the aging heart without ES cell injection (data not shown). Richly GFP-positive spots (Fig. 5C) were found during fluorescent microscopy in aging myocardium with cell injection. These GFP-positive spots suggest that engrafted ES

cells, or at least some of them, survived in the aging myocardium at 6 weeks after cell injection. There was no evidence of overt rejection, which is reflected by a lack of encapsulation and an absence of significant lymphocyte infiltration. The aging heart with cell transplantation showed positive staining to cardiac-specific α-MHC (Fig. 5D), including the area of newly regenerated cardiac tissue. Colocalization of engrafted ES cells with fluorescent staining for α-MHC is shown in Figure 5E. These data suggest that engrafted ES cells not only survived in the aging myocardium 6 weeks after cell injection but also differentiated into cardiac tissue.

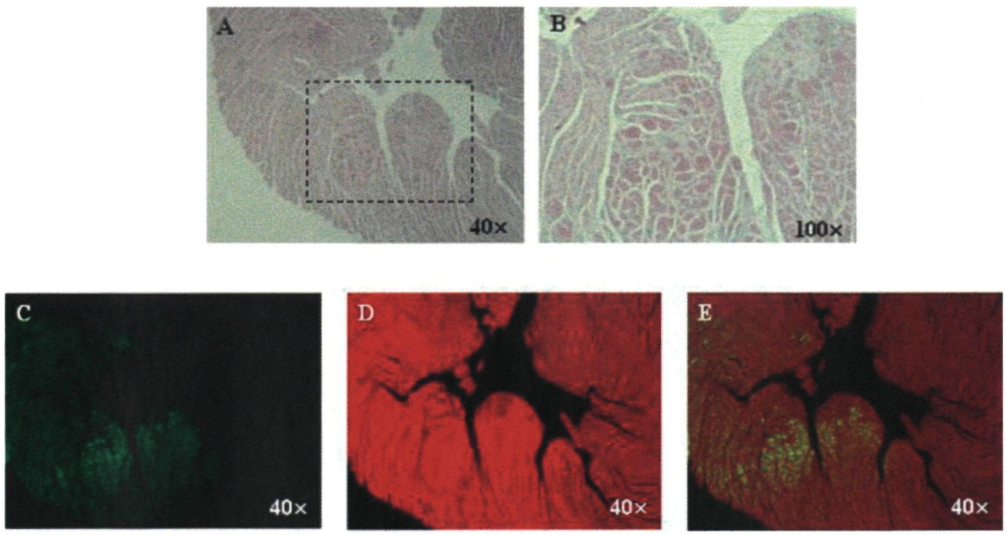

Figure 5. Panel **A** shows engrafted ES cells with hematoxylin and eosin staining in the aging myocardium 6 weeks after cell transplantation. Panel **B** shows the higher magnification image corresponding with the image in panel A pointed out by a dot-lined rectangle to show regenerated tissue from implanted ES cells. GFP-positive spots are shown within the aging host myocardium in panel **C**. The α-MHC staining of the myocardial section from the aging heart with cell transplantation, as shown in panel **D**, indicates the positive staining not only displayed in the host myocardium but also in the regenerated tissue. Panel **E** shows colocalization of transplanted ES cells by using fluorescent staining for cardiac α-MHC.

DISCUSSION

Advanced aging is associated with a decrease in cardiac contractility and relaxation [18,19]. Potential mechanisms include a significant loss of cardiac myocytes in animals [3] and in human subjects [5] as part of the normal aging process. Anversa and associates [3] reported a significant loss of myocytes in the left ventricles after advanced aging. A further study by Kajstura and colleagues [4] indicated that the progressive increase in apoptotic and necrotic myocyte death in the aging hearts of Fisher 344 rats was associated with the occurrence of ventricular dysfunction, which was apparent between 16 and 24 months of age. The results of the present study clearly demonstrate a significant myocyte loss in aging hearts from Fischer 344 rats at the age of 24 months compared with that seen in the hearts of 3-month-old rats. ES cell therapy at 6 weeks after transplantation restored the number of myocytes toward normal values. The survival of engrafted ES cells was confirmed by the finding of GFP-positive tissue in the aging myocardium 6 weeks after cell transplantation, which also had differentiated into cardiac tissue, as reflected by positive staining to cardiac-specific α-MHC. The positive response to β-adrenergic stimulation was blunted in the aging heart without ES cell treatment but could be partially restored 6 weeks after ES cell transplantation. This finding is consistent with our previous results with isolated papillary muscles in diseased hearts [11,17] and the results of others [20] with perfused aging hearts. The abnormal ventricular function and isoproterenol responsiveness in the aging heart might be attributed to the reduction in protein level of SERCA2, which is mainly responsible for the dysfunction of myocardial performance [17] in infarcted rat hearts. Previous studies showed a reduction in the content and activity of SERCA2 in the aging rat heart [21] and senescent human myocardium [22]. Loss of cardiomyocytes in the aging rat heart is the major factor contributing to the reduction of SERCA2 protein levels. Decrease in the protein levels of SERCA2 in the aging heart resulted in impaired intracellular Ca2+ handling, which is responsible for reduction of cardiac performance and attenuation of the inotropic effect to isoproterenol stimulation. Partial restoration of the inotropic responsiveness to isoproterenol stimulation by ES cell transplantation was related to increased protein levels of SERCA2. More experiments are needed to clarify the effects of ES cell therapy on the expression of major cardiac contraction proteins and intracellular Ca2+ handling in the aging and diseased aging hearts. In addition, β-receptor downregulation might also evolve into decreased isoproterenol stimulation responsiveness in the aging heart that cannot be excluded without further experimental testing.

Advanced age is accompanied by impaired angiogenesis in the vascular beds of the heart [6, 23] and peripheral tissues [24] at rest and during ischemic stress. Diminished angiogenesis in old rats and rabbits was the result of impaired endothelial function and a lower expression

of vascular endothelial growth factor in nonischemic and ischemic tissue [24]. With the isotope microsphere techniques, the present study demonstrated a significant reduction of the regional blood perfusion in the aging left ventricles. ES cell transplantation partially restored the regional blood flow of the left ventricle in the aging heart at 6 weeks after cell injection. This finding is consistent with our previous reports that showed stem cell transplantation could enhance angiogenesis by increasing neovascularization in postinfarcted rat myocardium [12] and increasing the left ventricular blood flow in postinfarcted porcine hearts [15]. New blood supply to the aging myocardium combined synergistically with regenerating cardiomyocytes derived from engrafted ES cells might prevent and reduce the pathogenesis of ventricular function associated with advancing age. It is possible that transplanted ES cells could release transcription signals (e.g. endothelial growth factor) to stimulate neovascularization in the surrounding tissue, or a portion of the engrafted ES cells might transdifferentiate into functional endothelial cells. Further experiments are required to understand the sophisticated mechanisms of blood flow improvement after cell transplantation. The linear regression analyses in the present study do not indicate a strong correlation between a single variable (either myocyte number or blood flow) and ventricular function. No single factor fully contributes all the benefits from cell transplantation in the aging hearts. Myogenesis and angiogenesis from engrafted ES cells provide synergistic effects leading to the improvement of cardiac function.

There are several limitations in the present study. First, cardiac function was measured in the unloaded condition. Preload, afterload, and heart rate might affect the functional difference between the young and aging hearts. Extensive systematic evaluation of cardiac function under the conditions of different loading conditions should be conducted in a future study.

Second, linear regression analyses might not reflect the natural feature of the host hearts after cell transplantation. The mechanism of cell therapy for cardiac repair is quite complicated and has not been fully addressed yet. Extensive explanation from regression analyses should be done cautiously because even a more complete multivariable regression study might not establish a causal relationship.

Third, myocyte increase after cell transplantation might not only result from engrafted ES cells per se but also from decrease of the apoptotic process in the aging myocardium caused by cardiac-protecting factors that were released from engrafted ES cells. Augmentation of angiogenesis results in an increase in left ventricular blood flow that might also contribute partially to a reduction in the process of apoptosis in the advanced aging heart.

Fourth, although there was no evidence of ventricular arrhythmia in the aging rat heart after ES cell transplantation with telemetric ECG monitoring, an extensive study should be performed to test cell therapy-associated arrhythmia before using this novel approach clinically in patients.

ACKNOWLEDGEMENTS

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CHAPTER 8

Summarizing discussion and concluding remarks

E. Kaplan

Stem cells provide us a promising future for the treatment of many diseases. A lot of basic and clinical stem cell research has been done; however, there are still many unanswered questions that need to be addressed before this promise can actually be realized in a broad patient population. In cardiovascular diseases, in particular the heart failure patients have a lot to gain by the prospect of generating new heart muscle. In our studies we tried to answer some of these remaining questions and focused on the issues concerning the mechanisms and methods behind stem cell delivery. These need to be addressed for stem cell therapy to become a potential strategy to treat heart diseases.

The aging heart

As we age, there are structural and functional changes in the heart and blood vessels. This is characterized by loss of cardiomyocytes, leading to loss of contractile mass and eventually impaired diastolic filling, diminished left ventricular systolic function, diminished contractility and increased arterial stiffness. The latter resulting in a raise in blood pressure [1,2]. The results of the experiments in **Chapter 7** also indicate a reduction of cardiomyocytes in aging rat hearts, leading the remaining myocytes to become hypertrophic as a coping mechanism. We determined that these hypertrophic myocytes were more prone to apoptosis; however, they were also more likely to fuse with and be rescued by stem cells (**Chapters 5 and 6**). Furthermore, our studies showed a decrease in blood perfusion in the aged hearts. Another confirmation of the decreased cardiac function in the aging heart is represented in our studies by a reduction of the LVSP and the increase in left ventricular end-diastolic pressure in aging rats. Available evidence suggests that the combination of these factors will lead to the development of heart failure.

Changes after myocardial infarction

Similar to the aging heart, a myocardial infarction also generates a loss of viable myocardium. The necrosis of myocytes results in several myocardial adaptations, such as wall dilatation, hypertrophy and scarring, in order to restore cardiac function. Wall dilatation and thinning of the infarcted area is the consequence of a combination of cell stretching, decrease in intercellular space and 'cell slippage', the rearrangement of myocytes after degradation of the intercellular collagen matrix (Fig. 1). The heart responses by hypertrophying myocytes to diminish progressive dilatation and increased load and stabilize contractile function [3-6]. These processes of remodeling may ultimately lead to systolic and diastolic dysfunction. In **Chapter 5**, we find similar results and show that apoptosis of cardiomyocytes significantly increased 3 days after MI. Also, we demonstrated in **Chapter 6** that angiotensin II, which is upregulated after MI, indeed hypertrophied myocytes and changed the calcium metabolism.

After myocardial infarction, several cytokines and adhesion molecules are released. We observed an upregulation of TNF-α and IL-6 and ICAM-1. We demonstrated that these factors play an important role in the homing and adhesion of stem cells after MI.

Myocarditis attracts stem cells

The importance of cytokines in cardiac diseases is also present in myocarditis, a disease that is characterized by inflammation of the heart and necrosis of cardiomyocytes. In **Chapter 3**, we demonstrated that several serum cytokine levels are elevated in myocarditis. In this thesis we conclude that tumor necrosis factor, one of the cytokines that was increased in our studies, plays a role in clearing the virus, as well as recruiting inflammatory cells and activating endothelial cells, resulting in the chronic phase in fibrosis, calcification and ultimately cardiomyopathy.

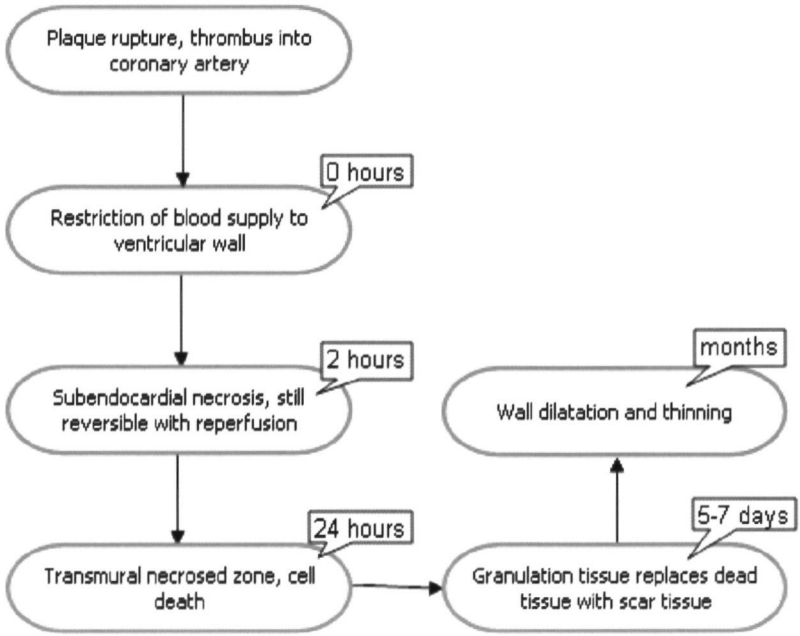

Figure 1. Summary of the pathophysiological effects after myocardial infarction.

Cell therapy improves cardiac function

In our studies (**Chapters 3-5 and 7**) we found that the damage inflicted by aging, infarction or inflammation can be partly reversed by cellular transplantation of stem cells. Improved cardiac structure and hemodynamic functions were demonstrated in the stem cell treated groups. In the presence of stem cells, the number apoptotic cardiomyocytes decreased, leaving more healthy numbers of cardiomyocytes. This resulted in less cardiac necrosis and hypertrophy. Injecting the stem cells when the heart was maximal inflamed and releasing the highest amount of cytokines, resulted in the largest decrease of necrosis. Left ventricular systolic pressure (LVSP) and the peak rate of the LVSP ($+dP/dt_{max}$) were significantly improved, the left ventricular end-diastolic pressure (LVEDP) decreased after stem cell infusion. The results of the experiments described in this thesis also demonstrate that cell therapy enhanced angiogenesis by increasing left ventricular myocardial blood perfusion and the numeric density of arterioles. This resulted in a higher 6-months survival rate of rats that received stem cell therapy.

Mechanisms of success

Different mechanisms have been described as the rationale behind the positive effects of stem cell transplantation. In order to discover the exact mechanism that is involved, we studied differentiation, fusion, anti-apoptosis and neoangiogenesis. For this, we used undifferentiated embryonic stem cell in our experiments as well as early-differentiated cells, where we initiated differentiation by using the hanging drop method (Fig. 2).

The embryonic stem cells were transfected with enhanced green fluorescent protein (GFP) before transplantation. In the studies described in **Chapters 3, 4 and 7**, most GFP spots, representing the survived infused stem cells, were found in myocardial tissue. The estimated area of GFP-positive spots in the myocardial sections constitutes about 5% of the total left ventricle. Differentiated stem cells into new cardiac-like tissue were confirmed by positive immunostaining for anti-cardiac troponin I antibodies in our first study (**Chapter 4**) and antibody immunostaining for cardiac-specific α-myosin heavy chain in our second study (**Chapter 7**). Moreover, in **Chapter 7**, we also measured cardiac protein levels of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2) in left ventricles, which is an indicator of the loss of cardiomyocytes, and found a restoration of SERCA2 levels after stem cell transplantation in a MI model. This suggests that the transplanted embryonic stem cells differentiated into cardiac tissue. After cell transplantation in aging rat hearts we found an increase in the number of total myocytes, which could be the result of transdifferentiation; however, this can also be the effect of other mechanisms, such as angiogenesis, fusion or anti-apoptosis.

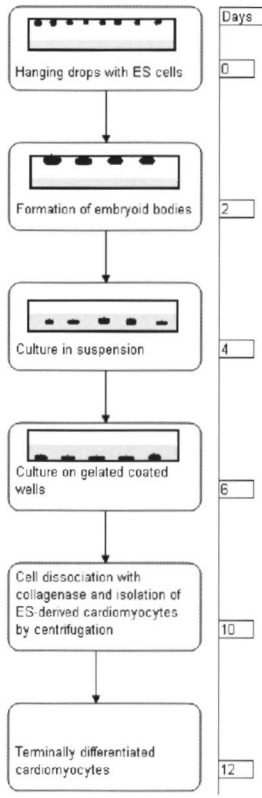

Figure 2. Schematic overview of the hanging drop method used to generate early-differentiated cells.

Recent studies have shown that cell fusion may result in the rescue of damaged myocytes otherwise destined for apoptosis or necrosis. Fusion cells have been demonstrated to form *in vitro* and *in vivo,* although relatively sparse in number. **Chapter 6** provides novel insights in the fusion process between myocytes and stem cells. We demonstrated that by inducing hypertrophy, the number of fusion events increased. Using Cre/lox recombination to detect fusion, we have shown that embryonic stem cells fuse with adult myocytes. Inducing hypertrophy by treating the cells with angiotensin II resulted in the reappearance of T-channels in adult myocytes, an increase in intracellular calcium and a depolarization of the membrane potential. These events contributed to the fusibility of the myocytes. Furthermore, treating the myocytes with the T-channel blocker amiloride, blocked hypertrophic adult myocyte fusion. In our studies we concluded that T channels are very important for the ability of myocytes to fuse with non-myocytes. Since T channels reappear after hypertrophy and MI, this could explain how fusion could play an important role in healing the sick heart with the help of non-myocytes.

As stated before, aging as well as damage to the heart muscle is associated with an increase in apoptotic myocytes and a loss of functional contractile mass We demonstrated that 3 days after myocardial infarction, apoptosis of cardiomyocytes significantly increased We examined the effect of stem cell therapy on myocyte apoptosis in an aging myocardial infarction model and found a decrease in the number of apoptotic cells after cell transplantation and an improvement in cardiac function and survival rate Furthermore, we found that the number of apoptotic myocytes decreased when co-cultured with embryonic stem cells in an in vitro hypoxia model These results indicate that preventing apoptosis could be the mechanism by which cardiac function is improved after cell transplantation

Increasing blood perfusion after myocardial infarction improves overall cardiac function In our study we showed that transplanting embryonic cells partially restored the regional blood flow of the left ventricle in the aging rat heart In the previous studies of our group we demonstrated similar results and found that stem cell therapy could enhance angiogenesis by increasing neovascularization in postinfarcted rat myocardium and increasing the left ventricular blood flow in postinfarcted porcine hearts The mechanism behind the angiogenesis is unclear, however, neovascularization could be explained by the release of growth factors by ES cells or by the transdifferentiation of ES cells into endothelial cells

Delivering stem cells to the heart

Successful transplantation of stem cells depends upon the migration of cells to the appropriate organ Two of our studies were performed using intravenous injection (through tail vein injection) of stem cells Cells delivered intravenously will less likely home to the heart, unless they are attracted to the heart and remain there long enough to proliferate Their migration has to be directed towards certain chemotactic agents in the heart TNF-α has proven to be a potent attractor of stem cells in our studies (**Chapters 2-4**) This is an important finding, as TNF-α is a cytokine that is released after cardiac damage and remains upregulated to 35 days after MI and could play an important factor in the timing and results of future stem cell transplantations in clinical studies In **Chapter 2** we describe how adding TNF-α to a Dunn chemotaxis chamber (Fig 3), resulted in the development of different membrane extensions that ensured migration towards the well filled with TNF-α TNF-α played a major role in the migratory behavior of stem cells, they migrated further, faster and in larger numbers

In **Chapter 4** we used transwell plates to demonstrate that cultured neonatal rat cardiomyocytes transfected with TNF-α cDNA significantly increased embryonic stem cells migration in comparison to cardiomyocytes without overexpression of TNF-α The third chapter of this thesis describes the effect of cytokines to stem cell homing and improvement

of heart function in animals with myocarditis. Fourteen days after virus inoculation mice displayed the highest serum levels of IL-6, IL-10, TNF-α and TNF-β. Mice that received the stem cell infusion at day 14 had the greatest improvement in hemodynamic performance and on necrosis. Another mechanism in stem cell homing that we found, was that calcium plays a central role in mediating stem cell movements. We developed a model to compute the mobility of stem cells, combining displacement, directionality and speed. Using this model, we compared different concentrations of calcium, cAMP and cGMP and their effect on stem cell migration and found that calcium is essential for stem cell migration. Increasing extracellular calcium resulted in a stimulation of cell migration and we found intracellular calcium levels to be upregulated during migration. The influence of calcium is associated with its effect on the actin cytoskeleton. The actin cytoskeleton is a complex of protein filaments that determines cell shape and movement. cAMP is also known for its impact on the organization of the actin cytoskeleton and we also found cAMP to increase the mobility of stem cells. However, cGMP has an inhibitory effect on cell migration. Its depressant effect is not explained in our studies, but it may be by suppressing and reorganizing the production of proteins that are needed in migration.

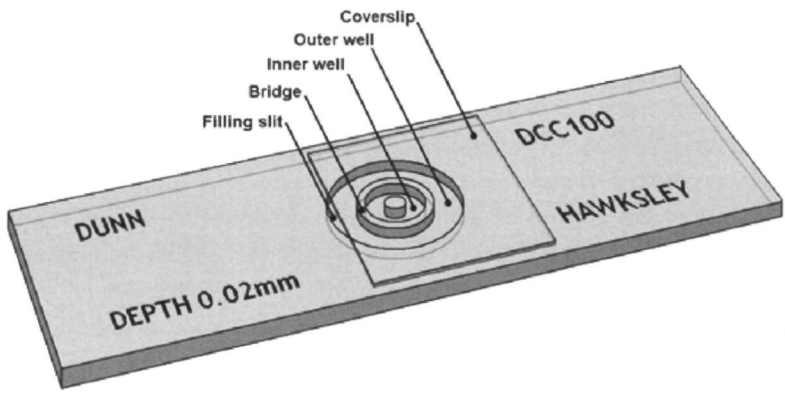

Figure 3. Dunn chemotaxis chamber. Copyright: Dunn.

In most studies stem cells were delivered through intramyocardial injection, with the advantage of trapping implanted cells in the selected injured areas of the heart In the experiments described in **Chapter 5 and 7**, we transplanted stem cells by intramyocardial injection Cells were injected in three different sites, one in the infarcted area and two in the bordering areas This resulted in improved cardiac functions Intramyocardial injection of cells could result in arrhythmias Therefore, we implanted an ECG transmitter in the rat abdominal cavity, but registered no significant cardiac arrhythmias

Conclusion and limitations

Although this thesis provides many novel insights into stem cell transplantation, there are some limitations In our second chapter we describe to be the first to show the second messenger pathways in undifferentiated embryonic stem cells However, transplanting undifferentiated stem cells possesses the risk of developing a teratoma For that reason, we did use early-differentiated cells in our animal studies Also, in our future studies the mechanism behind the effect of cAMP and cGMP on stem cell migration should be examined The results might be related to the intracellular calcium concentration, but there might be other factors that play a role In our third chapter we continue with the research on stem cell migration and find several factors that are released from myocardium during inflammation to be important for the homing of stem cells In that study we also showed the shortcoming of intravenous stem cell delivery, traces of stem cells were also detected in the liver, spleen and kidney of the mice In our fourth chapter we translated these findings into a myocardial infarction model and found that intravenously infused embryonic stem cell-derived cells homed to the infarcted heart, improved cardiac function, and enhanced regional blood flow after myocardial infarction We explained these results as being associated with locally released cytokines after myocardial infarction and the effect of transdifferentiation of stem cells The limitation of that study, as well as our aging study (**Chapter 7**), was the measurement of cardiac function in unloaded condition Different loading conditions might affect the results and therefore the analysis of the advantages of stem cell transplantation

We hypothesized that it is unlikely for transdifferentiation of stem cells to account for the vast cardiac improvements and therefore examined other mechanisms that might contribute such as anti-apoptosis, fusion and neovascularization In our fifth chapter we found that part of the cardiac improvement after stem cell transplantation is associated with the reduction of cardiomyocyte apoptosis This mechanism however did not completely explain the benefits of stem cell transplantation Also, to provide more evidence for apoptosis, we should use electron microscopy in future studies The following study described the mechanism of fusion and this resulted in the finding that hypertrophic myocytes fuse easily with stem cells However, these results were not confirmed in vivo studies We conclude our thesis with the

observation that stem cell transplantation in the aging heart results in the regeneration of cardiomyocytes and the increase of regional blood perfusion. More experiments are needed to explain the enhancement of blood perfusion as well as the improvement of cardiac function. In conclusion, we found that there are different mechanisms that contribute to the positive effect of stem cell transplantation, but that more research is needed before using this novel treatment in patients.

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CHAPTER 9

Summary in Dutch (Nederlandse samenvatting)

List of abbreviations

List of publications

Dankwoord

Curriculum vitae

E. Kaplan

Nederlandse samenvatting

Een acuut myocardinfarct (MI) zorgt voor veel morbiditeit en mortaliteit in de Westerse wereld en is de voornaamste veroorzaker van hartfalen. Na een acuut MI vinden er verschillende fysiologische processen plaats. In eerste instantie vindt er necrose van myocyten plaats, waarna de myocyten zich primair aanpassen door uitrekking en reorganisatie waardoor wanddilatatie van het myocard ontstaat en secundair hypertrofie om het verlies aan myocyten te compenseren waardoor wandverdikking optreedt.

Er is altijd aangenomen dat het hart niet het vermogen beschikt om te regenereren. Uit recente onderzoeken blijkt het hart echter over endogene stamcellen te beschikken en wel degelijk proliferatieve capaciteiten te beschikken. Dit is helaas niet voldoende om te compenseren voor de miljoenen myocyten die het hart verliest na een infarct. Er zijn inmiddels vele excellente behandelingen om verder functieverlies na een acuut MI te voorkomen, deze compenseren echter niet voor de aangedane schade aan de hartspier. De enige therapie waarbij dit wel mogelijk is, is stamcel transplantatie. Er zijn al vele onderzoeken gedaan naar deze baanbrekende therapie, er blijven echter veel vragen onbeantwoord. Deze vragen dienen eerst beantwoord te worden, voordat deze nieuwe benadering toegepast kan worden in de kliniek.

Dit proefschrift is geschreven met het doel succesvolle regeneratie van het hart te bereiken. Wij hebben ons gericht op twee speerpunten; het in kaart brengen van het mechanisme waarmee stamcel transplantatie werkt en de verschillende manieren om deze cellen te transporteren naar het hart.

Om deze doelen te bereiken zijn eerst experimenten uitgevoerd om de migratoire capaciteiten van embryonale stamcellen in kaart te brengen. Twee van de in vivo studies zijn uitgevoerd door stamcellen intraveneus bij muizen in te spuiten. In **Hoofdstuk 2** beschrijven wij hoe het toevoegen van TNF-α aan een chemotaxis kamer zorgt voor meer en langer migrerende stamcellen. Dit is relevant gezien het feit dat TNF-α tot 35 dagen na een myocardinfarct wordt afgegeven en dus belangrijk is bij de timing van stamcel therapie. Hierbij tonen wij eveneens aan dat calcium en cAMP een belangrijke rol spelen in het stimuleren van stamcel migratie en van belang zijn voor het "homing" proces van stamcellen.

In **Hoofdstuk 3** tonen we aan dat TNF-α eveneens een belangrijke cytokine is bij het ziektebeeld myocarditis. Door middel van een myocarditis diermodel demonstreren wij

migratie van intraveneus geinjecteerde en GFP-gelabelde stamcellen naar het ontstoken myocardweefsel. Dit resulteert na 90 dagen in een verbetering van de cardiale functies. Wij hebben GFP-positieve myocyten gelokaliseerd in het myocardweefsel, waarmee we aantonen dat de stamcellen naar het hart gemigreerd zijn en deze waarschijnlijk getransdifferentieerd zijn in myocyten.

De experimenten van **Hoofdstuk 4** bevestigen de voorgaande resultaten en wij demonstreren dat na inductie van een myocardinfarct in een diermodel, de geinjecteerde stamcellen naar het beschadigde hart 'homen' en 6 weken nadien zorgen voor een verbetering van de cardiale functies en de regionale bloedtoevoer. Wederom tonen wij GFP-gelabelde cellen in het myocardweefsel. Dat deze gedifferentieerde stamcellen over dezelfde kenmerken beschikken als myocyten wordt vastgesteld door positieve immunostaining voor anti-cardiac troponin I antilichamen. In deze studie vinden wij wederom bevestigd dat lokaal vrijgekomen cytokines na een myocardinfarct, zoals TNF-α, bijdragen aan het homingproces van stamcellen.

Aangezien het merendeel van hartfalen voorkomt in de oudere bevolking, hebben wij de effecten van veroudering op het hart getest als wel het effect van stamceltherapie. Experimenten in **Hoofdstuk 5** beschrijven dat door veroudering een vermindering van de totale hoeveelheid myocyten optreedt. De resterende myocyten reageren hierop door hypertofisch te worden en zijn daardoor gevoeliger voor apoptose. Wij tonen aan dat de hartfunctie van een verouderd hart wordt gekenmerkt door vermindering in perfusie, reductie van de linker ventrikel systolische druk en een toename van de linker ventrikel eind-diastolische druk. Het injecteren van stamcellen verbetert deze cardiale functies en zorgt eveneens voor een afname van het aantal apoptotische cellen en een langere overleving. Anti-apoptose is dus mogelijk een mechanisme waardoor de hartfunctie verbetert na stamcel-transplantatie.

Het volgende mechanisme dat in **Hoofdstuk 6** van dit proefschrift wordt beschreven is fusie. Celfusie houdt in dat de membranen van twee cellen met elkaar fuseren en een gezonde cel kan daarmee mogelijk de redding zijn van een beschadigde cel. Verschillende onderzoeken tonen echter dat fusie een weinig voorkomend mechanisme is. Onze experimenten beschrijven nieuwe inzichten op het gebied van fusie tussen myocyten en stamcellen. Wij demonstreren een hogere frequentie van fusie door het induceren van hypertrofie bij myocyten. Dit wordt gerealiseerd door de myocyten te behandelen met angiotensine II. Hierdoor worden de T-type calciumkanalen in myocyten gestimuleerd, het type kanaal dat eveneens zorgt voor de grote fuseerbaarheid van andere type cellen, zoals bijvoorbeeld myoblasten. Het belang van T-kanalen voor fusie wordt te meer bevestigd

doordat het blokkeren van het T-kanaal middels amiloride, resulteert in een significante afname van het aantal gefuseerde cellen.

In **Hoofdstuk 7** worden de experimenten beschreven van een MI diermodel bij het verouderde hart. Getransplanteerde stamcellen vinden wij gedifferentieerd terug in het beschadigde hart en dit wordt bevestigd door een positieve immunostaining voor "cardiac-specific α-myosin heavy chain". Eveneens constateren wij na stamcel transplantatie een toename van het eiwitgehalte van sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2) in de linker ventrikels; verlies van cardiomyocyten is de voornaamste oorzaak van een vermindering van de hoeveelheid SERCA2. Eveneens constateren wij na stamcel transplantatie een toename van het eiwitgehalte van sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2) in de linker ventrikels, een indicator van afname van cardiomyocyten. Het totaal aantal cardiomyocyten blijkt bij bepaling inderdaad toegenomen na stamcel transplantatie. In deze studie tonen wij vervolgens aan dat het transplanteren van embryonale stamcellen zorgt voor een herstel van regionale bloedflow in de linker ventrikel. Dit kan het effect zijn van transdifferentiatie van embryonale stamcellen in endotheliale cellen, of het uitscheiden van groeifactoren die de angiogenese stimuleren.

Concluderend stellen wij vast dat verschillende mechanismen een bijdrage leveren aan het positieve effect van stamcel transplantatie, maar dat meer onderzoeken nodig zijn voordat we deze nieuwe behandelwijze kunnen toepassen in een patiëntenpopulatie.

List of frequently used abbreviations

3α-MHC	alpha-myosin heavy chain
bFGF	basic fibroblast growth factor
BMDC	bone marrow derived stem cell
$[Ca^{2+}]_e$	extracellular calcium concentration
$[Ca^{2+}]_i$	intracellular calcium concentration
cAMP	cyclic adenosine 3,5-monophosphate
cGMP	cyclic guanosine 3-5'-monophosphate
cTNI	cardiac troponin I
$-dP/dt_{max}$	maximum rate of left ventricular systolic pressure decrease
dP/dt_{max}	maximum rate of left ventricular systolic pressure rise
EDC	embryonic stem cell derived cell
EMCV	encephalomyocarditis virus
ERK	extracellular signal-regulated kinases
ESC	embryonic stem cell
GAPDH	glyceraldehyde-phosphate dehydrogenase
GFP	green fluorescent protein
ip	intraperitoneally
LV	left ventricular
LVEDP	left ventricular end diastolic pressure
LVSP	left ventricular systolic pressure
MI	myocardial infarction
MMP	matrix metalloproteinase
NO	nitric oxide
PAF	platelet activating factor
SCT	stem cell transplantation
SERCA2	sarcoplasmic reticulum calcium ATPase
SDF-1α	stromal-cell-derived factor-1 alpha
T-channel	T-type calcium channel
TNF-α	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor
X-gal	5-bromo-4-chloro-3-indolyl-D-galactoside

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Curriculum vitae

The author of this thesis was born in Doetinchem, the Netherlands, on 3 June 1980. In 1991, she attended the Christelijk College Schaersvoorde in Aalten, where she graduated in 1997. At University of Utrecht, she started in 1997 with Sociology and Arabic, New Persian and Turkish languages and cultures. After finishing her propedeutical exam, she started medical school in 1998 at the Radboud University Nijmegen. As a medical student, she started working in 2003 as a research fellow at the Charles A. Dana Research Institute and the Harvard-Thorndike Laboratories, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts, United States. She was trained in basic research and worked on stem cell migratory patterns under supervision of principal investigator, Professor James Morgan. After half a year, she returned to the Netherlands to finish her Master's degree and instead of starting her clinical rotations as planned, she moved to Boston to pursue her PhD on stem cell therapy in the field of cardiology in a research fellowship monitored by her copromotor Professor James Morgan and supervised from Nijmegen, the Netherlands by Professor Freek Verheugt. In 2006 she returned to finish her clinical rotations at the University Medical Center Nijmegen and in the meantime finish writing this thesis. She passed her M.D. examinations in 2008 and after working as a cardiology resident in Nijmegen, she started working in July 2009 as a cardiology resident at the Isala klinieken, Zwolle, under supervision of dr. Anand R. Ramdat Misier.