Mesenchymal Stem Cells for Cellular Cardiomyoplasty: The Role of Anti-Inflammatory Cytokines

BY:

Guangyong Chen, MD

Divisions of Cardiac Surgery and Surgical Research

McGill University

Montreal, Quebec, Canada

August 2008

A thesis submitted to McGill University in partial fulfillment of therequirements of the degree of Master of Science in Experimental Surgery

Your file Votre référence
ISBN: 978-0-494-66906-8
Our file Notre référence
ISBN: 978-0-494-66906-8

PREFACE

The experiments leading to this thesis were all performed at the University Surgical and Laboratories of the Montreal General Hospital, McGill University Health Center and funded by a grant from the Fonds de la Recherche en Sante du Quebec (FRSQ).

This work has been or will be presented in the following meetings

(1) Anti-inflammatory Cytokines may play Salutary Paracrine Effects in Cellular Cardiomyoplasty using Mesenchymal Stem Cells (MSCs).

19th Annual Fraser N. Gurd Day, Department of Surgery, McGill University, Montreal, May 2008.

(2) The Potential Salutary Paracrine Effects of Anti-inflammatory Cytokines May Play a Role in Cellular Cardiomyoplasty Using Mesenchymal Stem Cells (MSCs)

2008 Canadian Cardiovascular Congress (CCC), Toronto, October 29, 2008

(3) The Potential Salutary Paracrine Effects of Anti-inflammatory Cytokines May Play a Role in Cellular Cardiomyoplasty Using Mesenchymal Stem Cells (MSCs)

American College of Cardiology 58th Annual Scientific Session, March 29, 2009, in Orlando, Florida, USA

In addition to presentations of original work, as a co-author I have published a paper as following.

R Atoui MD,M.Sc, J-F Asenjo MD, Minh Duong B.Sc, **G Chen** MD, Ray C-J Chiu1 MD, PhD and Dominique Shum-Tim MD,MS. Marrow Stromal Cells As "Universal Donor Cells" For Myocardial Regenerative Therapy: Their Unique Immune Tolerance. The Annals of Thoracic Surgery, 2008 Feb; 85 (2):571-9

ACKNOWLEDGMENTS

I would like to thank all people who encouraged, supported and helped me doing my two-year study.

I wish to express my most sincere gratitude and profound appreciation to my supervisor Dr. Dominique Shum-Tim for his continuous scientific guidance, encouragement and financial support. Without his help, I would not able to finish my master study.

I would like to thank Dr. Ray C-J Chiu. His understanding, incredible support and personal guidance and advice have provided a good basis and a great inspiration to me on this project. He has guided not only my graduate degree but also my academic and self-learning and self-improvement path.

I sincerely thank Mrs. Minh Duong for her help, experimental assistances, and useful suggestions. I would also like to thank Dr. Juan-Francisco Asenjo, Dr. Jianjun Chen, Madhur Nayan B.Sc, Dr. Adil Al Kindi, Yin Ge B.Sc, and Ming-Kai Ho for their help and wonderful friendship.

I am grateful to Mrs. Irene Sidorenko, the coordinator of graduate program in my department, for informing me all rules and requirements, Mrs. Allana Melanson and Mrs. Emma Lisi for making all of this possible

Finally, I want to thank all my family members for their understanding, support, encouragement and love.

This work is dedicated to my 6-month-old daughter, Yue Chen.

TABLE OF CONTENTS

PREFACE....2
ACKNOWLEDGEMENTS....3
TABLE OF CONTENTS....4
LIST OF FIGURES....7
ABSTRACT....9
RéSumé....10
Chapter I: Introduction and background....12
1. Background of infarct healing in the myocardial infarction(MI)....14
1.1. Repair process in healing infarcts....15
1.2. Cytokine release during MI and heart failure....16
1.3. The cytokine cascade....18
1.3.1. Interleukin-1(IL-1)....18
1.3.2. Interleukin-6 (IL6)....19
1.3.3. Interleukin-8 (IL8)....20
1.3.4 Interleukin-10(IL-10)....21
1.4 Pro-inflammatory/anti-inflammatory cytokine imbalance in acute coronary syndromes....23
1.5 Innate immune cells Mobilization Post-Myocardial Infarction....24
1.5.1 Cell-mediated inflammatory response....25
1.5.2 Neutrophils....25
1.5.3 Monocytes/macrophages....26
1.5.4 Mast cells....26
1.6 Repression of inflammatory gene expression and resolution of the inflammatory infiltrate....26
1.6.1 Clearance of apoptotic neutrophils....27
1.7 Extracellular matrix remodeling in the healing infarct: the role of the MMP2 and TIMP1....27
2. Myocardial regeneration and paracrine action....29
2.1 MSCs regeneration property....30
2.2 MSCs Immunomodulation propert....32

2.2.1 Mechanism of MSCs immunomodulation.....34
3. Clinical implications.....38
4. Conclusions.....39
References.....41
Chapter II: Material & Methods.....61
1. Study rationale.....62
2. Animal 63
3. Experimental design.....63
4. Isolation, culture, and labeling of rat MSCs.....63
5. X-gal staining for detection of ß-galactosidase activity.....64
6. Preparation of cells for injection.....65
7. Creation of the Infarction and Intramyocardial Transplantation of MSCs.....65
8. Cardiac Functional Assessment.....66
9. Hearts harvest and Tissue Processing.....66
10. Total RNA Preparation and First-Strand cDNA Synthesis.....67
11. Quantitative Real-time PCR.....67
12. RT-PCR and Gel Bands semi-quantification.....69
13. Histopathological analysis.....70
14. Collagen Content and Area measurement.....70
15. Statistical Analysis.....71
Chapter III: Results.....72
1. MSCs Culture.....73
2. Mortality and Sample Size.....74
3. Inflammatory Cytokines Gene Expression Changed in Left Ventricle after MSCs-Treatment 74
4. MMP2 and TIMP1 Gene Expression in LV.....80
5. ECM Deposition Measurement.....82
6. Histopathological Observation and Myocardial Injury Score.....85
7. Cardiac Functional Assessment.....87
Chapter IV: Discussion.....90
Chapter V: Conclusions.....95

References.....97

LIST OF FIGURES

Figure 1. Spindle-shape appearance of MSCs in a culture dish.....73
Figure 2. X-gal staining of labeled MSCs in culture flasks.....73
Figure 3. Comparative Cytokines Gene expression in LV treated with MSCs at 12 Hours.....75
Figure 4. Comparative Cytokines Gene expression in LV treated with MSCs at 24 Hours.....76
Figure 5. Comparative Cytokines Gene expression in LV treated with MSCs at 1 Week.....76
Figure 6. Comparative Cytokines Gene expression in LV treated with MSCs at 2 Weeks.....77
Figure 7. Comparative Cytokines Gene Expression Changes in Group C with Times.....77
Figure 8. Pro-/anti- inflammatory Cytokines Gene Expression Ratio at 12 Hours.....78
Figure 9. Pro-/anti- inflammatory Cytokines Gene Expression Ratio at 24 Hours.....78
Figure 10. Pro-/anti- inflammatory Cytokines Gene Expression Ratio at 1 Week.....79
Figure 11. Pro-/anti- inflammatory Cytokines Gene Expression Ratio at 2 Weekrs....79
Figure 12. Pro-/anti-inflammatory Cytokines Gene Expression Ratio Changes with Times.....80
Figure 13 Gene expression of MMP2 and TIMP1 in LV at various times after MSCs implantation.....81
Figure 14. MMP2 Gene Expression Changes with Times.....81
Figure 15. TIMP1 Gene Eexpression Changes with Times.....82
Figure 16. MMP2/TIMP1 Gene Expression Ratio Changes with Times.....82
Figure 17. Picture scanned from a slide of a rat heart suffered from left ascending artery ligation for 2 weeks, under the staining of Sirius red.....83
Figure 18. Infarct scar area and the total area of LV myocardium measured automatically by the computer by the means of ImageJ-1.41 (NIH, US).....84

Figure 19. ECM Area of Left Ventricle.....85
Figure 20. Representative sections(stained with H&E and X-gal) of rat.....86
Figure 21. Tissue examination for acute myocardial injury and inflammatory cell infiltration at 12 and 24 hours 87
Figure 22. Representative M-mode echocardiogram performed 2 weeks after implantation(C) 88
Figure 23. Ejection fractions at various times 89
Figure 24. Fractional shortening at various times 89

ABSTRACT

BACKGROUND Adult bone marrow derived MSCs had been explored to treat myocardial infarction (MI) and heart failure, for which various beneficial paracrine effects had been suggested. Since MSCs in vitro express anti-inflammatory cytokines, we tested the hypothesis that changes in the pro-/anti-inflammatory cytokine ratio in the infarct microenvironment may provide such a paracrine mechanism to improve early cardiac function following acute coronary occlusion.

Methods Rats (n=88) underwent acute left coronary artery ligations and were randomized into groups M and C and then injected with culture media or MSCs, respectively. These rats underwent blinded echocardiography to evaluate left ventricular ejection fractions (LVEF). Real Time PCR was used to compare cytokine gene expression for IL-1β, IL-6, IL-8 (pro-inflammatory) and IL-10 (anti-inflammatory) at various times. Extra-cellular matrix (ECM) deposition and inflammatory cell infiltration were also analyzed.

Results As early as 12 hours, the ratio of pro-/anti-inflammatory cytokine gene expression in group C was significantly lower than group M. Similar results were found at 24 hours,1 and 2 weeks, respectively. LVEF improved significantly in group C (M=62% vs C=68% at 12 hours*, M=66% vs C=75% at 24 hours*, M=57% vs C=75% at 1 week*, and M=52% vs C=70% at 2 weeks*, *$p<0.01$). The ratio of MMP-2/TIMP1 levels was lower in the Group C at all time frames, reaching significance at 12 and 24 hours and 2 weeks. In group C, histopathological analysis revealed significantly less ECM deposition (M=1.95% vs C=0.75% at 24 hours*, M=19.30% vs C=9.36% at 1 week*, M=24.46% vs C=7.57% at 2 weeks*, *$p<0.01$). This was associated with significantly decreased inflammatory cell infiltration after 24 hours.

Conclusions The current data suggests that MSCs therapy decreases the pro-/anti-inflammatory cytokine ratio in the infarct microenvironment. This is associated with improved cardiac function, reduced ECM deposition, and decreased inflammatory cell infiltration. This paracrine mechanism of MSCs therapy may explain the early functional improvement after MI before cell transdifferentiation or other mechanisms takes place.

Résumé

INTRODUCTION Les cellules souches mésenchymales (MSC) dérivées de la moelle osseuse adulte ont été étudiées pour traiter les infarctus myocardiques et les arrêts cardiaques, pour lesquels divers effets paracrines bénéfiques ont été suggérés. Puisque les MSC in vitro expriment des cytokines anti-inflammatoires, nous avons testé l'hypothèse comme quoi des changements au niveau du ratio de cytokines pro-/anti-inflammatoires dans le microenvironnement de l'infarctus pourraient mettre en marche le mécanisme paracrine qui améliorerait la fonction cardiaque immédiate (à court terme) suivant une occlusion coronaire aiguë

Méthodes Des rats (n=88) ont subi des ligations aiguës de l'artère coronaire gauche et ont été placés au hasard dans les groupes identifiés M et C. Ceux du premier groupe ont reçu une injection de milieu de culture, alors que ceux du second en ont eu une de MSC. Ces rats ont subi une échocardiographie pour évacuer les fractions d'éjection ventriculaire gauche (FEVG). Le test de PCR en temps réel a été utilisé pour comparer l'expression des gènes pour IL-1β, IL-6, IL-8 (pro-inflammatoires) et IL-10 (anti-inflammatoire) sur des laps de temps différents. Les dépôts de matrice extra-cellulaire (MEC) et l'infiltration des cellules inflammatoires ont aussi été analysés.

Résultats Après seulement 12 heures, le ratio d'expression du gène des cytokines pro-/anti-inflammatoires du groupe C était beaucoup plus bas que celui du groupe M. Des résultats similaires furent notés après 24 heures, 1 semaine et 2 semaines. Les FEVG se sont améliorées grandement dans le groupe C (M=62% vs C=68% après 12 heures*, M=66% vs C=75% après 24 heures*, M=57% vs C=75% après 1 semaine* et M=52% vs C=70% après 2 semaines*, *p<0.01). Le ratio des niveaux de MMP-2/TIMP1 était moins élevé dans le groupe C à chaque laps de temps, le résultat devenant significatif après 12 et 24 heures, ainsi qu'après 2 semaines. Dans le groupe C, une analyse histopathologique a révélé des dépôts beaucoup moins importants (M=1.95% vs C=0.75% après 24 heures*, M=19.30% vs C=9.36% après 1 semaine*, M=24.46% vs C=7.57% après 2 semaines*, *p<0.01). Ce phénomène était associé à une infiltration des cellules inflammatoires beaucoup moins grande après 24 heures.

Conclusions Les données présentées ici laissent paraître que la thérapie aux cellules souches mésenchymales (MSC) diminue le ratio de cytokines pro-/anti-inflammatoires dans le microenvironnement de l'infarctus. Ce phénomène est associé à une amélioration de la fonction cardiaque, une diminution du dépôt de matrice extra-cellulaire et une réduction de l'infiltration des cellules inflammatoires. Ce mécanisme paracrine de thérapie par cellules souches mésenchymales pourrait expliquer l'amélioration rapide de la fonction cardiaque après un infarctus myocardique avant qu'une transdifférenciation cellulaire ou que tout autre mécanisme ait lieu.

Chapter I

INTRODUCTION AND BACKGROUND

With the advances in diagnostic techniques and pharmacological, interventional and surgical therapies, the survival rates in patients with cardiovascular diseases have improved. As a result, congestive heart failure (CHF) becomes the only cardiovascular disorder that is increasing in incidence over time in North America. Heart failure is now the most common cause of hospitalization in persons over 65 years old and has increased by 155% during the past 20 years [1]. Heart failure constitutes a public health problem as an emerging epidemic. Approximately 5 million patients were diagnosed to have CHF in United States alone and 550,000 new cases are diagnosed yearly [2,3]. At least a tenth of this prevalence is expected to exist in Canada. This represents a chronic and progressive disease characterized by frequent hospitalization and high annual mortality rates (20-40%)[4]. In addition, patients with CHF have demonstrated a much poorer quality of life compared to patients with other chronic diseases, scoring poorly on various physical function, emotional well-being and overall social scales [5, 6]. Current therapies for CHF are associated with limited success, inherent complications and significant cost without addressing the fundamental pathophysiological problem related to the loss of functioning contractile cardiomyocytes. Therefore, there is a need to look for new therapies.

A novel approach currently under intensive investigation is cellular transplantation, which is directly aimed to overcome the problem of myocardial cell loss. We first introduced the term 'cellular cardiomyoplasty in 1995 to indicate this new therapeutic strategy consisting of replacing dead cardiomyocytes with newly functional contracting cells [7]. This method consists of transplanting cells into the infracted area of the myocardium to 1) increase or preserve the number of cardiomyocytes, 2) to improve vascular supply, and 3) to augment the contractile function of the injured myocardium. Since its introduction, animal studies and human clinical trials have shown that bone marrow derived marrow stromal cells (MSCs) implantations have significant clinical benefits in patients with myocardial infarction (MI) and CHF [8-10]. However, despite its promising initial results, this technology is perceived with skepticism. This is mostly because of its still early stage of development, controversial mechanistic insight, contradiction to dogmatic principles etc.

In this introduction, we will focus on the role of marrow stromal cells in the process of inflammatory response during the heart repairing and myocardial regeneration, their

effects in various experimental models of myocardial injury and we will review the most updated information regarding the cellular and molecular signaling mechanisms through paracrine effects of these MSCs administrated into the injury site and then undergo 'milieu-dependent' or in situ differentiation. Furthermore, we will introduce the recent concept of MSCs immunomodulation and explore the evidence of different mechanisms proposed for property.

1. Background of infarct healing in the myocardial infarction (MI)

Inflammation is an essential component in the pathogenesis of much common cardiovascular disease, including myocardial infarction (MI), congestive heart failure, and generalized atherosclerosis. The involvement of the inflammatory mechanism in heart disease is still poorly understood and continues to challenge scientists and clinicians. The role of inflammation is a natural evolutionary response to injury, and a mechanism for healing and tissue repair. However, the inflammatory response can often be inadequate, or overwhelming, leading to direct accelerated injury and even death of the host [11]. A greater understanding of the delicate control system of the inflammatory cascade in heart disease would be important for identifying key mechanisms in the disease process, and aid in the development of new therapeutic approaches.

During acute myocardial infarction and other acute coronary disease, there are presently several inflammatory proteins, cytokines, chemokines, and other intercellular adhesion molecules, that have been identified and studied as potential markers for development of various cardiovascular disease such as myocardial infarction and heart failure. MI is associated with an intense inflammatory response and activation of the host innate immune program for inflammatory cell mobilization and cardiac repair and remodeling. The repair process post-MI begins with an acute inflammatory response beginning ar 12-16 hours after the onset of ischemia. Neutrophilic granylocytes involving in removing dead myocytes migrate into the infarcted region first and then peak at 24 to 48 hours post-infarct [12]. Then macrophages and lymphocytes follow and help clear up the cellular debris. Subsequently the granulation tissue forms the border zone of the infarct region.The remodeling and repair of the infarct area take place 2-3 weeks to a year after MI. For the whole remodeling process, the inflammatory response causes self-repair and

protection. However if there is an excessive autoimmune inflammatory response, due to extreme cytokine and chemokine productions, a large number of immune cells will be mobilized to the infarcted heart and this will lead to faster remodeling and hypertrophy [13].

1.1 Repair process in healing infarcts

Sudden induction of ischemia by coronary artery occlusion triggers a series of events that culminates in the death of ischemic cardiomyocytes [14]. Cardiomyocyte necrosis trigger an inflammatory cascade that serves to clear the infarct of dead cells and matrix debris, but also results in healing and replacement of the damaged tissue with scar. Thus, cardiac repair after myocardial infarction is closely intertwined with the inflammatory response.

The myocardial healing process can be schematically divided into four distinct phases [15,16]. (i) Necrotic phase: acute cell death secondary to necrosis and apoptosis (immediately after MI). (ii) Acute inflammatory phase: inflammatory response in order to absorb necrotic tissue (1–7 days). During the inflammatory phase, activation of chemokine and cytokine cascades results in recruitment of leukocytes into the infarcted area. Neutrophils and macrophages clear the wound of dead cells and matrix debris. (iii) Sub-acute granulation phase: Activated macrophages release cytokines and growth factors, leading to the formation of granulation tissue. At this stage, expression of inflammatory mediators is suppressed, whereas fibroblasts and endothelial cells proliferate. Granulation tissue formation consisting of proliferating myofibroblasts, which increase the myocardial tensile strength and promote blood vessel proliferation thereby improving tissue perfusion and cell survival (1–3 weeks). (iv) Chronic scar phase: fibroblasts formation and micro-vessel regression generating the final collagen- rich scar tissue (>1 month). Fibroblasts and vascular cells undergo apoptosis, and a collagen-based scar is formed

Infarct healing results in profound changes in ventricular architecture and geometry also referred to as "ventricular remodeling". The molecular and cellular changes associated with ventricular remodeling affect both the cardiomyocytes and interstitial cells and manifest clinically as increased ventricular size, altered shape of the ventricle, and worsened cardiac function. Remodeling is linked to heart-failure progression and is

associated with poor prognosis after myocardial infarction. Ventricular dilation after myocardial infarction is an important predictor of mortality [17]and adverse cardiac events (35), including the development of heart failure and ventricular arrhythmias [18,19].

Although the pathways involved in remodeling remain poorly understood, it is clear that the pathologic and structural changes associated with infarct healing directly influence remodeling and affect prognosis in patients with myocardial infarction. After cardiomyocytes die in the infarcted myocardium, granulation tissue cells and the extracellular matrix network provide mechanical stability to the injured tissue. Thus, preservation of the collagenous matrix is important to minimize infarct expansion. In addition, defects in the healing process may be directly involved in the development of lethal complications, such as cardiac rupture and ventricular aneurysm formation. Cardiac rupture involves tearing of acutely infarcted tissue and results from mechanical weakening that occurs in the necrotic and inflamed myocardium.

1.2 Cytokine release during MI and heart failure

MI triggers a series of cellular and molecular mechanisms that regulate inflammation. Rapid induction of innate immune response leads to local intense release of cytokines like tumor necrosis factor (TNF-α) [20], IL-1ß et al. Research has shown that cytokines such as TNF-α is elaborated intrinsically within the myocytes within minutes of ischemia or structural injury. One major pathway that may coordinate the host injury response following injury such as infarction is the innate immunity-inflammaory cytokine and chemokine activation pathways, which have been reviewed recently. These stress cytokines include paracrine factors such as angiotensin, aldosterone, endothelin, and TNF-α, IL-1ß, and IL-6 et al. These inflammatory cytokines secreted post-MI in turn promote signal transduction pathways leading to growth factor and chemokine production such as stem cell factor (SCF), Flt-3/Flk-2 Ligand (FL), Stromal cell-derived factor-1 (SDF1) and matrix metalloproteinase (e.g. MMP9), which permits cell migration, shape change and phenotypic alterations, along with deposition of abnormal collagen scar elaboration remodeling factors [21,22].

The cytokine inflammatory response post-MI can have two opposing activities, one as a chemoattractant in order to mobilize innate immune cells to the myocardium that protect against infection or the spread of cellular apoptosis and damaged tissue. However, cytokine activity can inversely influence the outcome by causing to more cardiovascular injury by altering myocyte contraction and inducing alteration of beta-adrenergic receptor signaling leading to apoptosis and myocyte hypertrophy.

Also, these inflammatory cytokines secreted post-MI, in turn, promote signal transduction pathways leading to growth factor and chemokine production such as SCF, FL, SDF1 and matrix metaloproteinases(e.g MMP9) [23], which permits cell migration, shape change and phenotypic alterations, along with deposition of abnormal collagen scar elaboration remodling factors. Therefore early cytokines mediated mechanisms are involved and are detrimental for cardiac repair post-MI by mediating intracellular signaling, inflammatory cell mobilization, as well as activation of growth factors and chemokine activity, setting a vicious cycle of cell loss-wall stress and structural and functional deterioration.

Ischemia acts as a part of the host stress response, leading to cytokine induction through various signalling pathways [24]. These pathways are up-regulated in response to diverse stimuli such as free radical excess, hypoxia, osmotic dysregulation, and early membrane injury. Peroxisome proliferator-activated receptor-g (PPAR-g) is another stress induced inflammatory regulator. Some PPAR-g agonists such as pioglitazone and rosiglitazone were associated with significant reductions in inflammatory cytokine levels in the myocardium and were found to improve myocardial function and remodelling [25].

Pro-inflammatory cytokines, such as TNF-α, IL-1ß, and IL-6, are produced as a stress response against myocardial injury [27]. These cytokines are not constitutively expressed in the normal heart [27, 28]. However within the first hours of ischemic injury, intramyocardial cytokines are up-regulated. The triggers of cytokine release during MI include ischemic stimulus, mechanical stretch, reactive oxygen species (ROS), and cytokine self-amplification pathways. If the infarction is large, or if host inflammatory response is exuberant, there can be either sustained cytokine up-regulation or a second wave of cytokine up-regulation, which may last for several weeks, corresponding to chronic remodelling phase. Cécile et al reported that in the infarcted region, TNF-α, IL-1ß, and IL-6 gene expression had been found peaked at 1 week after infarction and

decreased rapidly thereafter. In contrast, at 20 weeks after infarction, the gene expression levels of these cytokines remained significantly higher in the noninfarcted than in the infarcted zone [29]. Furthermore, the levels of these cytokines in the noninfarcted region correlated with the left ventricular end-diastolic diameter measured at 8 and 20 weeks after infarction (G1).

1.3 The cytokine cascade

Numerous studies have demonstrated activation of cytokine cascades in the infarcted myocardium. Induction and release of the proinflammatory cytokines TNF-α, IL-1ß, and IL-6, IL-8 are consistently found in experimental models of myocardial infarction [30,31,32]. Complement activation, free radical generation, and NF-KB activation are capable of stimulating cytokine mRNA synthesis in both resident and blood-derived cells, resulting in marked cytokine upregulation in the infarcted area. One of the characteristic features of cytokines is their functional pleiotropy and redundancy: one cytokine exhibits a wide range of biologic effects on various cell types, and similar cytokines exert similar and overlapping actions on the same cell type [33]. The multifunctional, overlapping, and often contradictory effects of the cytokines have hampered understanding of their functional role in infarct healing. Although the exact roleand interaction of these cytokines in mediation of the injury is not clear, extensive charactization of their physiological role has been described in the literature.

1.3.1 Interleukin-1 (IL-1)

The IL-1 gene family consists of three members: IL-1a, IL-1ß, and IL-1 receptor antagonist (IL-1Ra). IL-1a and IL-1ß are agonists, whereas IL-1Ra is a specific receptor antagonist [34]. Both IL-1a and IL-1ß are capable of inducing the expression of other cytokines, chemokines, growth factors, and adhesion molecules. Marked IL-1ß upregulation has been reported in experimental models as well as patients with myocardial infarction [35, 36, and 37].

In the infarcted region, IL-1ß gene expression had been found peaked at 1 week after infarction and decreased rapidly thereafter. In contrast, at 20 weeks after infarction, the gene expression levels of these cytokines remained significantly higher in the

noninfarcted than in the infarcted zone [38].Another study showed that the peak of IL-1β gene expression was 24 h later after ligation.

Another investigation suggested a protective role for IL-1ß demonstrating that IL-1ß neutralization in the acute phase of myocardial infarction resulted in increased occurrence of cardiac rupture and enhanced adverse remodeling [38]. Much like TNF-α, IL-1ß also modulates fibroblast phenotype inducing AT-1 receptor expression [39] and upregulating matrix metalloproteinase synthesis [40]. As a highly pleiotropic and multifunctional mediator, IL-1 is likely to have a wide range of actions on various cell types involved in the healing response.

1.3.2 Interleukin-6 (IL-6)

IL-6 is a member of a larger family of structurally related cytokines with overlapping biologic effects. Extensive experimental evidence demonstrated induction of members of the IL-6 family in healing infarcts [41]. IL-6 synthesis is rapidly induced in mononuclear cells and cardiomyocytes of the ischemic myocardium [42,43,44]. Cardiac myocytes are also reported to produce IL-6 under hypoxic stress [45]. IL-6 has profound effects on cardiac myocytes by promoting cardiac hypertrophy, but also by protecting them from apoptosis [46].

IL-6 is capable of modulating the phenotypic characteristics and gene expression of many cell types involved in infarct healing. An increasing number of experimental observations suggest that IL-6 is also capable of modulating cardiovascular function, exerting a negative inotropic effects. Mice with overexpression of both IL-6 and IL-6 receptors have been reported to show constitutive tyrosine phosphorylation of gp130 and to develop cardiac hypertrophy [47]. IL-6–null mice demonstrated significantly delayed cutaneous wound healing, suggesting a significant role for IL-6 in tissue repair [11].

However, Fuchs and co-workers [48] found that the absence of IL-6 did not affect infarct size, left ventricular function, and postinfarction remodeling in nonreperfused infarcts. Although these findings do not preclude biologically significant actions of IL-6 in healing infarcts, it appears that in mice lacking IL-6, other mediators may act in a compensatory manner to activate the JAK/STAT pathway, thereby maintaining STAT3 phosphorylation, which is crucial for the cellular effects of IL-6–related cytokines.

IL-6 is an acute reactant cytokine with very early expression of the infracted myocardium. Serum level of IL-6 is elevated after MI, and the myocardium is the major site of IL-6 production during myocardial ischemia. IL-6 delays the apoptosis process in neutrophils with greater capacity for oxidant production. Il-6 also has a regulatory role in the generation of other cytokines such as IL-8, IL-1ß. The effects of IL-6 on neutrophils are postulated to play a role in the mechanisms whereby IL-6 contributes to multiple organ dysfunctions.

Both mRNA and protein levels of TNF-α and IL-6 are increased within 15-50 min of left anterior descending artery (LAD) occlusion in the heart homogenates, and these elevated level are generally sustained for 3 hours, then cytokine mRNA's levels all returned to baseline levels at 24 h, while IL-1ß, TNF-α and TGF-b1 mRNA levels again rose significantly at 7 days in animals with LAD occlusion [49]. In another study, IL-6 gene expression peaked at 6 h and its expression remained higher at 2 days post-MI after permanent ligation [50].

1.3.3 Interleukin-8 (IL-8)

IL-8 is a chemokine produced by macrophages and other cell types such as epithelial cells and endothelial cells.

IL-8 is a potent chemoattractant for neutrophil. It can activate neutrophils as well as T lymphocytes, and control their trafficking. IL-8 is induced by the ischemic myocardium in animal models [51, 52]. A considerable body of clinical evidence has also documented that the myocardium is a major source of IL-8 during reperfusion after long duration of ischemia [53], or after acute myocardial infarction (AMI) [53,54], and IL-8 peaked with 29.6-fold difference ligation and gradually declined thereafter to control levels at 7 days post-MI [64]. The release of both superoxide and IL-8 were higher in patients with complicated AMI than in those with uncomplicated AMI, indicating IL-8 is a major contributor to the priming of neutrophils and may subsequently enhance the extent of injury [55]. Correlated with the reduced expression of IL-8, neutrophil influx into the infarcted area was decreased in C6-deficient rabbits subjected to regional ischemia and reperfusion [56].

In as much as neutrophil activation is a critical initial step in ischemia-reperfusion injury, it has become evident that administration of anti-IL8 antibodies in rabbits prevents cardiopulmonary injury [57, 58, and 59]. Indeed, a strong correlation between IL-8 production and postoperative cardiac troponin-I levels has been observed [60]. It should be noted that, in addition to IL-8, other pathways exist for the recruitment of neutrophils to the inflammatory site. For instance, circulating neutrophils from patients undergoing aortic aneurysmectomy show delayed expression of constitutive apoptosis and such a prolonged neutrophil survival may contribute to the inflammatory injury [61]. Nevertheless, IL-8 blockade has no direct effect on neutrophil apoptosis [62]. Interestingly, IL-8 also has a function of angiogenesis, which is protective for the diseased heart [63].

1.3.4 Interleukin-10 (IL-10)

IL-10, a cytokine predominantly expressed by activated Th2 lymphocytes and stimulated monocytes, possesses potent antiinflammatory properties [65,66]. Among the different cell types affected by IL-10, monocyte–macrophages appear to be particularly modified in regard to their function, morphology, and phenotype.

The potential role of IL-10 in experimental myocardial infarction has recently been explored (Fig. 1) [67, 68]. IL-10 inhibits the production of IL-1a, IL-1ß, TNF-α, IL-6, and IL-8 by lipopolysaccharide activated monocytes, suppressing the inflammatory response. Furthermore, IL-10 may play a significant role in extracellular matrix remodeling by promoting tissue inhibitor of metalloproteinases (TIMP)-1 synthesis, leading to stabilization of the matrix [69]. This effect was inhibited when the incubation contained a neutralizing anti–IL-10 antibody. IL-10_/_ mice showed an enhanced inflammatory response after myocardial infarction demonstrated by increased neutrophil recruitment, elevated plasma levels of TNF-α [66]. These studies underscore the importance of IL-10 in the healing process, suggesting that IL-10 may be involved in inhibition of the postinfarction inflammatory response and in extracellular matrix remodeling

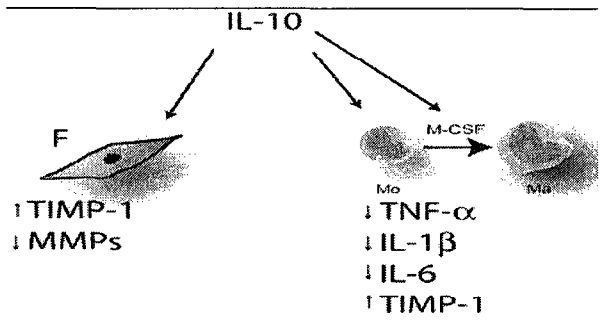


Figure 1 IL-10 may play an important role in infarct healing by suppressing expression of monocyte-derived inflammatory cytokines and by promoting extracellular matrix accumulation through enhanced TIMP synthesis. In addition, IL-10 enhances M-CSF–mediated monocyte (Mo)-tomacrophage (Ma) differentiation. F, fibroblast [190].

IL-10 mRNA and protein upregulation was demonstrated in the reperfused infarcted myocardium by using a canine model of myocardial infarction. IL-10 expression was first detected at 5 hours and peaked after 96–120 hours of reperfusion, while it has also been reported that IL- 10 expression peaked at 7 days instead of 2 days post-MI and was back at control level at 28 days post-MI [67].

It is noteworthy that cytokines act both individually and within a network of interrelated and interacting signals. IL-10 production is often proportional to the release of IL-8 during clinical CPB. For instance, the release of IL-10 during off-pump CABG is lower than in conventional procedure, which could have been related to the reduced production of IL-8 [70]. In combination with the reduction of IL-6 and IL-8, reduced production of IL-10 has also been associated with the use of heparin-coated CPB circuits in patients undergoing heart and heart-lung transplantation [71]. Furthermore, this was associated with reduced postoperative myocardial injury as reflected by a lower release of cardiac troponin-I 12 and 24 hours after reperfusion [72]. Thus, keeping a balance between pro- and anti-inflammatory reactions, instead of blocking some individual mediators, may be more crucial in determining the extent of the inflammatory response and the clinical outcome.

However, recently an in vitro experiment showed that IL-10 did not alter pro-inflammatory chemokine synthesis by cardiac fibroblasts. This suggested that IL-10 signaling plays a non-critical role in suppression of inflammatory mediators, resolution of the inflammatory response, and fibrous tissue deposition following myocardial infarction. This study examining the effects of several cytokines in tandem and comparing the pro-inflammatory ones, namely IFN-gama and TNF-α, to anti-inflammatory ones, namely IL-10, has shown that patients with acute MI or unstable angina demonstrate increased level of anti-inflammatory mediators in blood and cell cultures, and a reduced level of pro-inflammatory IL-10. This demonstrates the cytokines imbalance that occurs due to acute coronary syndromes.

1.4 Pro-inflammatory/anti-inflammatory cytokine imbalance in acute coronary syndromes

Collectively, studies over the past year have strengthened the association between inflammation and acute coronary syndromes disease. Tissue destruction acute coronary syndromes are associated with the presence of pro-inflammatory cytokines, such as TNF-α, IL-1ß, IL-6 and IL-8 [73, 74]. However, it has also been suggested that pro-inflammatory cytokines can activate homeostatic mechanisms to suppress inflammation [75]. This dual activity of many key cytokines involved in acute coronary syndromes has been a key topic recently, and understanding these mechanisms will be necessary to understand the different inflammatory cytokines expression profile.

The inflammatory process is mediated through a complex cytokine network. The clinical expressions and outcome of the disease can vary among different ethnic groups, possibly depending upon the differential expressions of MHC and cytokine genes. Cytokines are protein messengers that convey information between and within cells via specific cell surface receptor molecules. The release of specific cytokines into the systemic circulation has been observed in a variety of inflammatory disease including MI. Their concentration levels usually reflect disease severity and prognosis.

However, most cytokines are expressed transiently, moreever different cytokines expression have their own peak time. These cytokines can be induced or inhibited by

other cytokines, so it has been suggested that a "cytokine network" may exist in which cytokines regulate each other [76].

In MI, the balance between pro-inflammatory and anti-inflammatory cytokines determines the degree and extent of inflammation, and thus can lead to different clinical effects. Anti-inflammatory cytokines or cytokine antagonists counteract the effects of pro-inflammatory cytokines and therefore the relative concentration of a cytokine to its inhibitor or antagonist will determine its final effect.

Proinflammatory to anti-inflammatory cytokine ratios can signal the balance between proinflammatory and anti-inflammatory forces. Previous studies [50] have demonstrated early induction of IL-6 mRNA (peaking at 3 hours of reperfusion, Ref. 24), which was markedly enhanced during the first 6 hours by reperfusion of the previously ischemic myocardium; however, by 24 hours, IL-6 induction was also observed in nonreperfused infarcts. Some current experiments suggested that IL-6 mRNA expression, which is maximal at 3 hours of reperfusion, is down-regulated after 24 hours of reperfusion in the same ischemic segments in which IL-10 mRNA induction is found. In contrast, animals with permanent occlusion showed minimal IL-10 mRNA up-regulation and high IL-6 mRNA expression, which persisted for 96 h [77]. IL-10 expression was first detected at 5 hours and peaked after 96–120 hours of reperfusion. These findings showed the temporal discrepancies for the cytokines expression. Thus, considering the balance between pro- and anti-inflammatory reactions may be more crucial in determining the extent of the inflammatory response and the clinical outcome.

1.5 Innate immune cells Mobilization Post-Myocardial Infarction

The body's immune system is a complex network of specialized cells and organs that define the body against foreign particles such as bacteria, viruses, and parasites. It has the ability to distinguish between self and non-self antigens as well as build up memory and become immune responsive to future exposure. By the process of hematopoiesis, which is dependent on growth and mobilization receptors such as c-Kit, Flt3 and CXCR4, the immune system produces and stores a great number of different cell types, including lymphocytes(white blood cells; B and T-cells), Natural Killer(NK) cells, Dendritic cells(DC) and macrophages. B-cells normally work by interacting with antigen presenting

cells as macrophages and DC, to produce and secret antibodies tagging specific substance to be destroyed by T-cells.

There are two types of T-cells, cytotoxic T-cells, which can identify foreign substance and work without B-cell antibodies to destroy them, and B-cell dependent T-cells, which required an antibody for tagging the foreign substance. Macrophages are versatile cells that can act as antigen presenters for T-cells to initiate immune response and inflammation or can act as scavengers by secreting powerful monokines and destroying old useless cells and debris. DCs are similar to macrophages in that they are antigen presenters; however they can also cross talk with NK and T-cells to signal destruction. The branch of the immune system that identifies foreigners and tissue or organ damage, is referred to as the innate immune system, whereas the branch that requires previous exposure and development of memory cells is referred to as acquired immune system.

Macrophages, NK cells, and DCs are the body's most powerful innate immunity cells and are the first line of defense in the bone marrow, spleen, and the circulation. The innate immunity players are always ready to stress caused by either infection or injury. NK cell, DCs, and macrophages not only response to a wide variety of host stresses quickly, but they process the antigen to be recognized by B-cells and prepare for the acquired immunity.

1.5.1 Cell-mediated inflammatory response

Myocardial inflammation is histological characterized by tissular accumulation of circulating leukocytes. Once stimulated by the vascular endothelium, they migrate through the vessel wall into the ischemic myocardial region. The adhesion of leukocytes to the damaged vascular endothelium results in a massive migration of these inflammatory cells (e.g. neutrophils, monocytes, mast cells) into the ischemic myocardium, thus increasing myocardial tissue inflammation. This endothelial adhesion and trans-vascular migration process may be favored by spontaneous or percutaneous coronary intervention (PCI) -induced coronary plaque rupture [78].

1.5.2 Neutrophils

Neutrophils' infiltration into the myocardial ischemic regions may increase the infarction size by promoting tissue inflammation and by their direct entrapment in the capillary micro-vessels, both leading to a reduced myocardial local perfusion [78,79]. Furthermore, local neutrophils' accumulation may also increase thromboxan B2 (TxB2) or other toxic substances production, which may lead to local vasoconstriction and platelet aggregation [80]. These phenomena are frequently present after PCI and may be co-responsible for the 'no reflow' syndrome observed in the AMI setting.

1.5.3 Monocytes/macrophages

After migration of monocytes into the myocardial tissue, they become active macrophages, which may release a variety of inflammatory substances, cytokines, and growth factors [81,82]. Intravascular circulating monocytes as well as macrophages contained in atheromatous plaques actively participate in the post-ACS or post-PCI inflammatory healing process.

1.5.4 Mast cells

Mast cells are stimulated by the complement 5a (C5a) adenosine-reactive oxygen complex, which is a necessary element for the development of the myocardial stunning process often observed in a post-ischemic setting. Mast cells are therefore actively implicated also in the post-MI inflammatory response. In fact, by degradation, they may release vascular endothelium growing factors, fibroblast growing factors, and histamine, all promoting elements of myocardial fibrosis and neoangiogenesis. During this fibrotic angiogenetic process, myofibroblasts contribute to the extra-cellular matrix and the neo-vessel formation aiming to constitute the final myocardial scar tissue [80,84].

1.6 Repression of inflammatory gene expression and resolution of the inflammatory infiltrate

Chemokine induction, cytokine release, and leukocyte infiltration are prominent events in the inflammatory phase of myocardial infarction and play a crucial role in phagocytotic removal of dead cells and debris. However, this acute localized inflammatory response is transient and is followed by resolution of the inflammatory infiltrate and fibrous tissue

deposition [85]. A crucial commitment is made during the late stages of the inflammatory phase to convert the response from phagocytosis and clearance of dead cells and debris to a mode that promotes tissue repair and scar formation [86]. Inhibition of chemokine and cytokine synthesis after a dramatic early peak is crucial for the repair process, preventing prolonged expression of inflammatory mediators in the healing infarct and suppressing continuous leukocyte recruitment and injury. Thus, optimal healing requires mechanisms inhibiting chemokine and cytokine synthesis, resulting in resolution of the inflammatory infiltrate and transition to fibrous tissue deposition. These mechanisms involve (a) clearance of the neutrophilic infiltrate, (b) inhibition of cytokine and chemokine synthesis, (c) removal of the fibrin-based provisional matrix, and (d) activation of fibroblasts and collagen deposition. Although very few studies have dealt with the process of resolution of inflammation in the healing infarct, understanding these concepts is crucial for planning strategies targeting the postinfarction inflammatory response.

1.6.1 Clearance of apoptotic neutrophils

Clearance of the granulocytic infiltrate by professional phagocytes is a prerequisite for resolution of the inflammatory process. Apoptosis is the predominant mechanism that determines the functional longevity of neutrophils in inflamed and infarcted tissues [87]. Apoptotic neutrophils are recognized and phagocytosed by macrophages in a process that involves macrophage CD44 ligation [88, 89]. Ingestion of apoptotic cells by macrophages results in synthesis and release of TGF-b, a mediator crucial for resolution of inflammation [90]. In contrast, phagocytosis of necrotic cells leads to release of proinflammatory mediators, resulting in persistent inflammation [91]. The relevance of these concepts in myocardial infarct healing remains unknown. However, timely clearance of the leukocytic infiltrate may play a crucial role in suppression of the postinfarction inflammatory response.

1.7 Extracellular matrix remodeling in the healing infarct: the role of the MMP2 and TIMP1

Extracellular matrix remodeling in the healing infarct is orchestrated by a family of zinc-containing endoproteinases, termed MMPs [92]. The growing list of MMPs includes

more than 20 human members with common functional features [93]. Gelatinases (MMP-2 and -9) digest gelatin and various other matrix proteins, such as collagens type IV (an important component of basement membranes, V, and XI, and laminin). MMPs are secreted in a latent proform and require activation for proteolytic activity.

Most MMPs are expressed in normal adult tissues. In the normal heart, latent MMPs are expressed in fibroblasts and cardiomyocytes and are distributed throughout the cardiac interstitium [94]. Early upregulation of MMP expression and activity is found in healing myocardial infarcts [95]. Changes in the cytokine milieu are likely to play a key role in regulating MMP synthesis by inflammatory cells and fibroblasts infiltrating the infarcted myocardium. IL-1ß and TNF-α increase MMP-2 and MMP-9 activity [96], whereas IL-10 and TGF-a enhance TIMP synthesis, promoting extracellular matrix accumulation [97,98]. MMP expression may play a role in regulating many cellular processes involved in infarct healing, including leukocyte migration, angiogenesis, degradation of the extracellular matrix, and remodeling. MMP processing also regulates the activity of cytokines and growth factors [99] and may inhibit chemokine-mediated effects by generating CC chemokine sreceptor antagonists with antiinflammatory properties [100]. Studies using MMP inhibitors and genetically targeted animals have demonstrated the importance of MMP-mediated matrix degradation in infarct healing and in the pathogenesis of left ventricular remodeling. Treatment with a broadspectrum MMP inhibitor significantly attenuated left ventricular dilation after myocardial infarction [101]. Inhibition of the MMP system is currently explored by several laboratories as a strategy with potential therapeutic implications in myocardial infarction [101,102,103]. Investigations using mice with targeted gene disruptions of specific members of the MMP family have significantly contributed to our understanding of the role of MMPs in infarct healing. MMP-9_/_ mice exhibited delayed healing after myocardial infarction, associated with reduced leukocyte influx into the infarct, and were protected from myocardial rupture [105]. In another independent study, mice with targeted deletion of the MMP-9 gene had attenuated postinfarction remodeling and decreased collagen accumulation in the myocardium [106]. Two studies demonstrated that MMP- 2_/_ animals were protected from early rupture and showed decreased adverse outcome

[107,108]. These effects were associated with reduced macrophage infiltration and delayed clearance of dead cardiomyocytes from the infarcted area [108].

MMP activity is controlled at three distinct levels: (a) transcription, (b) activation of proMMPs, and (c) inhibition by the TIMPs MMP degrade matrix components and are inhibited by specific inhibitors, termed tissue inhibitors of metalloproteinases (TIMPs). The balance between MMPs and TIMPs is critical for remodeling of the extracellular matrix [109]. TIMPs also may play an important role in regulating extracellular matrix remodeling after myocardial infarction. In the absence of injury, TIMP-1_/_ mice have increased left ventricular end-diastolic volume in association with a decrease in myocardial collagen content, suggesting a role for TIMP-1 in maintaining normal left ventricular structure and geometry [110]. After myocardial infarction, TIMP-1_/_ mice exhibited accelerated left ventricular remodeling [111] that was pharmacologically "rescued" by MMP inhibition [112]. In contrast, TIMP-1 overexpression in the infracted heart resulted in complete prevention of cardiac rupture [113]. These findings suggest a protective role of TIMP-1 in infarct healing and left ventricular remodeling.

2. Myocardial Regeneration and Paracrine Action

Despite significant advances in medical and surgical management of heart failure, mostly of ischemic origin, the mortality and morbidity associated with it continue to be high. Pluripotent stem cells are being evaluated for treatment of heart failure. Emerging evidence suggests that locally delivered mesenchymal stem cells (MSCs) can lead to an improvement in ventricular function, but the cellular and molecular mechanisms involved remain unclear. A lot of investigations and clinical trials are presently underway to define the exact role of cell therapy in the management of heart failure. Emerging evidence suggests that bone marrow stem cells can lead to a reduction in the size of the myocardial scar, but the exact mechanism by which they act in the ischemic heart remains unknown [114,115]. There are plenty of questions that remain unanswered. Which cell type will be the most appropriate? Will it be more beneficial to inject a specific subpopulation of bone marrow cells rather than injecting unfractionated bone marrow? When to inject after myocardial infarction and where to inject into the scar tissue? The ultimate fate of these injected stem cells is also not known. Do they really form new

myocardial fibers or is the improvement in ventricular function cytokine mediated? [115] Even if they lead to regeneration of myocardial tissues, there is no consensus on whether the newly formed tissues integrate with the host tissues and contract synchronously.

A world of the literature suggests that attempts have been made to answer these questions. A variety of cell types like bone marrow cells [118,119,120], skeletal myoblasts [121,122], embryonic stem cells [120,125,117], cultured cardiomyocytes [123,124], have been used.

2.1 MSCs Regeneration Property

Bone marrow-derived mesenchymal stem cells (MSCs) have been extensively studied. Bone marrow contains stem cells of nonhaematopoietic tissues, currently referred to as mesenchymal stem cells (MSCs) because of their ability to produce cells of mesenchymal origin [125]. They have also been called marrow stromal cells as they appear to arise from a complex array of supporting structures found in the marrow [125]. MSCs were first characterized by Friedenstein and Petrokova [126]. Haynesworth et al. [127] were able to isolate and culture MSCs in therapeutic quantities. MSCs have been isolated from other sources like adipose tissue, skeletal muscle, human deciduous teeth and trabecular bone [128].

Bone marrow is the most common source of MSCs and has been well characterized in terms of surface markers and differentiation pathways [128]. MSCs represent only 0.001% of the nucleated cells in the bone marrow [129]. They do not express CD34 or CD45 and have been characterized phenotypically as nonhaematopoietic cells [130]. In fact, there are no specific cell surface markers, though they stain positive for CD29, CD44, CD105 and CD106 [131]. They are spindle-shaped and look like fibroblasts in their undifferentiated state [126,132,133,134,135]. They can differentiate into tissues of mesenchymal origin [126,133,136]. They can be expanded in cultures and have unique immunologic properties [135]. The primary goal of therapy for patients with acute myocardial infarction is rapid revascularization of ischemic tissues. Some clinical and animal studies have shown that bone marrow cells have the potential to produce tissue revascularization and regeneration, but the cellular and molecular mechanisms involved are still unknown [116,117].

Bone marrow contains several primitive pluripotent cells. At present it is difficult to point out which cell type will be most suitable to produce nonhaematopoietic tissues. Orlic et al. [141] injected Lin-c-kit+ cells in myocardial infarcts produced in experimental rats. These are bone marrow cells that express early cardiac lineage markers. Equivalent cells in humans are CD34+/AC133+ cells found in bone marrow mononuclear cell fraction. This experimental study showed positive results in terms of cardiomyocyte regeneration. Improvement in ventricular function was seen, but the mechanism of it remains unanswered as they could not show integration with the host tissues, communication with the host tissues (formation of gap junctions and intercalated discs) or synchronous contraction. There was no electron microscopic study involved. Sakai et al. [142] compared three cell types (fetal cardiomyocytes, smooth muscle cells and fibroblasts) in an attempt to elucidate the mechanism by which the fetal cardiomyocytes, when implanted in myocardial scar, lead to an improvement in myocardial function. They showed an improvement in ventricular function in those injected with fetal cardiomyocytes only, but failed to answer questions like how the cells survived in scar tissue. Where did the blood supply come from?

Findings from experimental studies suggesting that bone marrow cells delivered locally can generate de-novo myocardium gained interest for their potential therapeutic role in coronary artery disease [140,153,154,155]. Researchers have also raised questions over whether stem cells actually differentiate into functioning myocytes and whether the number of engrafted cells could account for the improved function. The small number of engrafted cells rules out increased myocardial mass as a mechanism for improved cardiac function [162]. In a rodent model of acute renal failure suffered from ischemia-reperfusion injury, significant renal function has recovered within 3 days after MSCs therapy. During this period, none of the administered MSCs had differentiated into any kidney tubular or endothelial cell phenotype. However, in the cell treated group, the inflammatory cytokines expression had significant changes, suggesting that the beneficial effects of MSCs are primarily mediated via complex paracrine actions [163]. However, stem cells prevent cardiac remodeling by some paracrine actions and some as yet undefined mechanisms [162].Tang et al. [156] raised serious doubts over myocardial regeneration leading to functional recovery. Very few engrafted MSCs express specific

cardiac markers, like connexin-43 and cardiac troponin I. Besides only limited gap junctions are formed between grafted and native cells. They have attributed the beneficial effects to the secretion of a number of angiogenic factors by the injected stem cells and the resulting paracrine effects [157,158]. Engrafted MSCs secrete chemokines like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which promote angiogenesis, attenuate apoptotic pathway, reduce left ventricular remodeling and improve cardiac performance [158]. Several other authors have favored the paracrine action theory rather than myocardial regeneration [159,160]. Nishida et al. [161] have shown that improved cardiac performance seen after bone marrow cell injection therapy is mainly due to increased blood flow. Angiopoietin-I and VEGF are the two most potent growth factors for inducing angiogenesis. They concluded that even if some cardiomyocyte differentiation occurred from these pluripotent cells, it would be insufficient to cause any improvement in cardiac performance [161].

Stamm et al. [143] and Galinanes et al. [144] demonstrated improvement in ventricular function following delivery of unpurified bone marrow into the scarred myocardium. Most of these studies have been carried out in conjunction with surgical revascularization, making interpretation of results difficult. Besides, unpurified bone marrow mononuclear cell suspension contains large numbers of leucocytes and their progenitors, which can induce local inflammation and cytokine mediated actions, rendering actual stem cell effects insignificant [145].

Studies have suggested that vascular trauma causes release of chemokines that causes selective recruitment of bone marrow-derived cells to the injured site, leading to organ revascularization and regeneration [146]. A number of chemokines/cytokines have been proposed to be involved in this mechanism [140,147,148,149]. Using daily injections of colony stimulating factor, Orlic et al. [141] showed improvement in animal myocardial infarction model. This finding was substantiated by other researchers [147,150], but several other studies could not reproduce the results [151,152].

2.2 MSCs Immunomodulation Property

Studies have shown that unrelated (allogeneic) MSCs are not immunogenic but rather immunosuppressive, which has huge clinical implications. These features of MSCs have

prompted researchers to investigate their role in cellular therapy [135,136]. They have a potential role in the repair of damaged tissues and in immunomodulation as well [135,136,137,138]. The full potential of 'regenerative medicine' can be realized only if we are able to use allogeneic cells. The ability of MSCs to avoid allogeneic rejection makes them ideal cells for this purpose [139].

Orlic et al. [140] transplanted bone marrow obtained from male mice into female mice and were able to show that newly formed cardiomyocytes were derived from injected bone marrow cells. Presence of Y chromosomes in the newly formed cardiomyocytes confirmed their origin from donor bone marrow cells. Failure to reconstitute infarcts was attributed to the difficulty of transplanting cells into tissues contracting at 600 bpm. They have acknowledged that an immunological reaction to the histocompatibility antigen on the Y chromosome or an unknown mechanism causing cell destruction cannot be ruled out.

When injected into damaged hearts MSCs will be exposed to inflammatory cytokines as we mentioned above and the host immune response. It is therefore important to also study human leukocyte antigen (HLA) expression and immunogenicity of MSCs [164]. A lot has been written about host immune response to embryonic stem cells and consequent need for systemic immunosupppression [165]. Not enough work has been done on immune interactions involving allogeneic bone marrow cells. McIntosh and Bartholomew [163] were the first to suggest that MSCs have immunomodulatory properties.

The critical question of host response to implanted MSCs is receiving increasing attention in recent times [128,133]. Important aspects of implanted cell–host interaction include the host immune response, the homing mechanisms and the differentiation of implanted cells under the influence of local signals. Studies have shown that MSCs are capable of suppressing T-lymphocyte proliferation in a mixed lymphocyte reaction (MLR) involving autologous or allogeneic T cells or dendritic cells (DCs) [166,167,168]. Di Nicola et al. [167] have shown that human T-cell proliferation, stimulated by the addition of allogeneic peripheral blood lymphocytes, DCs or phytohaemagglutinin, was suppressed by addition of MSCs in the cultures. The inhibitory effect is not restricted by major histocompatibility complex (MHC) because it occurs irrespective of the species of MSCs

or T cells [169]. This suggests that MSCs can be transplanted between MHC-incompatible individuals [139].

Emerging evidence suggests that human MSCs, because of their distinct immunophenotypic profile associated with absence of HLA class II expression and low expression of costimulatory molecules, may be nonimmunogenic or hypoimmunogenic [138]. This property may eventually allow use of allogeneic MSCs in the acute setting, e.g. following acute myocardial infarction. One potential disadvantage, as with any allogeneic approach, is the risk of disease transmission from donor to recipient. Using a baboon skin graft model Bartholomew et al. [170] have shown evidence of immunosuppression by MSCs in vivo.

MSCs are not lysed by alloreactive natural killer (NK) cells [171]. They inhibit the formation of cytotoxic T lymphocytes, mediators of graft-versus-host disease (GVHD) [172,173]. MSCs inhibit T cells only when they are present in culture and the effect is reversed when MSCs are removed. It indicates that the inhibitory effects are transient and not persistent [174]. Inhibitory effects of MSCs do not get modified during expansion in cell culture or even after irradiation [129].

MSCs have been used clinically for their immunomodulatory potential [175].

2.2.1 Mechanism of MSCs immunomodulation

How MSCs avoid allogeneic rejection is a matter of debate. They are hypoimmunogenic and do not express MHC-II. They do not express costimulatory molecules and perhaps, therefore, do not activate alloreactive T cells [166,167,168]. They prevent T-cell responses through modulation of DCs and NK cells [174,176]. Comparisons have been made to mechanisms involved in immune evasion by fetal allograft and tumor cells [177,178,179].

Several mechanisms have been proposed for the immunomodulatory action, namely apoptosis[129], soluble factors mediated [166,167,174,176], cell-to-cell contact [167,174,180,181], tryptophan depletion [182], and direct modulation of DCs, CD4+, CD8+ and NK cell function [174,176,183,184].The exact mechanism underlying immunomodulatory effects of MSCs remains unclear. Plumas et al. [129] are of the view that they inhibit T-cell proliferation by inducing apoptosis. Research by other

investigators, however, suggests that T cells do not become apoptotic as they can be stimulated again if MSCs are removed [167,168]. Some researchers are of the opinion that it represents a specific suppression of MLR, and is not due to apoptosis [128,117].

MSCs express HLA class I molecules, so they can be recognized as targets by preactivated alloreactive cytotoxic T lymphocytes, but they suppress differentiation of cytotoxic T-lymphocyte precursors into cytotoxic T-lymphocyte effectors through secretion of suppressive factors [183]. Analysis of cytokines in suppressed MLR cultures demonstrates up-regulation of interferon-[gamma] (IFN-[gamma]) and interleukin (IL)-10, and down-regulation of tumor necrosis factor-[alpha] production relative to control cultures [185]. Thus, the immunosuppressive effect appears to be partially mediated by soluble factors, although data are conflicting [167,168,169,174]. MSCs in culture do secrete several factors like IL-6, IL-8, prostaglandin E2 (PGE2) and VEGF [185]. Krampera et al. [183] have suggested the role of soluble inhibitory factors, but Plumas et al. [129] did not find any evidence to support this theory. According to Di Nicola et al. [167] suppression of T-lymphocyte proliferation by MSCs is mediated by soluble factors including hepatocyte growth factor (HGF) and transforming growth factor-[beta]1 (TGF-[beta]1). Besides, they also found that this antiproliferative effect was reversed by the addition of monoclonal antibodies against TGF-[beta]1 and HGF [167]. The role of HGF is controversial, with some studies reporting that addition of anti-HGF monoclonal antibody to MLR in presence of MSCs restored T-lymphocyte proliferation, while a recent study has shown that anti-HGF monoclonal antibody failed to restore T-lymphocyte proliferation [175]. Several other investigators failed to detect any of these molecules (HGF, TGF-[beta]1, IL-10 or IL-4) in MLR supernatant [169,17458,63].

Another possible mechanism is the production of PGE2 by MSCs, which can mediate their immunosuppressive effects [175]. PGE2 is known to modulate a variety of immune functions. Aggarwal and Pittenger [176] have shown that inhibiting production of PGE2 leads to reversal of MSC-induced immunomodulatory effects. Tse et al. [167] have shown that MSC production of PGE2, IL-10, TGF-[beta]1 or tryptophan depletion of culture medium were not responsible for the immunosuppressive effects. Similarly, Puissant et al. [169] are of the view that IL-10, TGF-[beta], and HGF are not involved in immunosuppressive effects. These contradictory results might be due to the use of MSCs

derived by different methods, variations in culture conditions, doses, and kinetics, which affect different cytokines and chemokines [160]. Thus, all the molecules tested so far to explain the MSC immunosuppressive properties are probably not involved. The soluble factor implicated remains to be defined.

Some researchers have shown that the inhibitory effect remains even when MSCs are separated from T cells by a semi-permeable membrane, indicating that it is mediated by soluble factors and cell-to-cell contact is not necessary [166,167]. Puissant et al. [180], on the other hand, have shown that when MSCs are separated from lymphocytes by a permeable membrane, the inhibitory effect gets impaired. They concluded that cell-to-cell contact is necessary for a full inhibitory effect. Similarly, Krampera et al. [174] have shown that the inhibitory effect is transient, dose-dependent and entirely dependent on the presence of MSCs, therefore, indicating that cell-to-cell contact is necessary. Djouad et al. [168] are of the view that cell-to-cell contact is necessary only if the ratio of MSCs/splenocytes is low.

In contrast to the findings of Di Nicola et al. [166], Maitra et al. [170] did not find MSC culture supernatant to have a T-cell inhibitory effect. Cell-free culture supernatant of MSCs failed to inhibit T-cell activation and, in fact, had a stimulatory effect on mixed lymphocyte cultures. In contrast, the cell-free supernatant obtained from a mixture of MSCs and unrelated lymphocytes had an inhibitory effect [170]. Some researchers have suggested that MSC-induced depletion of tryptophan in the culture medium could be the possible mechanism [171]. Indoleamine 2, 3-dioxygenase mediated conversion of tryptophan to kynurenine has been suggested as T-cell inhibitory effectors pathway [171]. The inhibitory effect of MSCs is not modified during expansion in culture. They modulate the immune system not only by a direct action on T cells but also at the very first step of the immune response through the inhibition of differentiation and maturation of DCs, the most potent antigen presenting cells in the body [186]. MSCs alter the cytokine secretion profile of DCs, naive T cells, and NK cells to induce a tolerant phenotype [176]. They inhibit secretion of potent proinflammatory cytokines, like TNF-[alpha] and IFN-[gamma], and stimulate secretion of suppressive cytokines, like IL-4 and IL-10 (176, Figure 2).

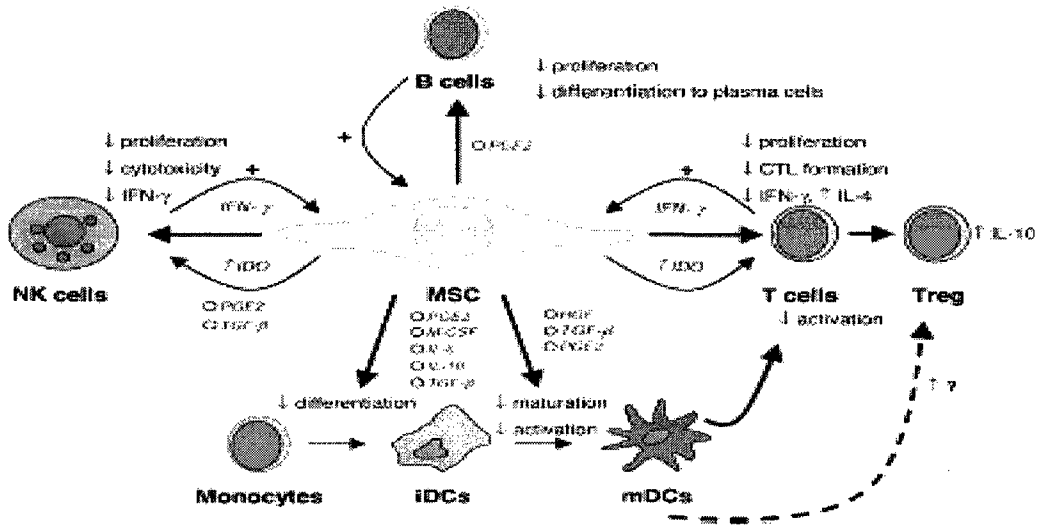


Figure 2. Immumomodulatory effects of MSCs. CTL indicates cytoxic T cell; HGF. Hepatocyte growth factor; IDO, indoleamine 2,3- dioxygenase; PGE2, prostaglandin E2; and TGF-ß, transforming growth factor ß. (Blood, 15 November 2007, Vol. 110, No. 10, pp. 3499-3506.)

The inhibition of secretion of tumor necrosis factor-[alpha] by DCs inhibits their maturation and migration [176,172]. Several other investigators have confirmed the influence of MSCs on the maturation and function of DCs [187]. MSCs also alter the phenotype and cause suppression of NK cells. Diverse mechanisms are involved in these suppressive functions. Some of these functions require cell-to-cell contact, while others are mediated by soluble factors like TGF-[beta]1 and PGE2 [187].

Several mechanisms are probably involved in MSC-induced immunosuppression depending on the lymphocyte population used, technique of MSC culture used and the type of stimulus used [135]. Le Blanc and Ringden [134] have suggested a nonspecific antiproliferative effect for MSCs as they suppress T-cell proliferation stimulated by both allogeneic antigens and mitogens such as phytohaemagglutinin and concanavalin A. Others have suggested a role of galectin-1 in the immunomodulatory actions of MSCs [188]. Galectin-1 is one of the major proteins expressed in MSCs and its expression is not affected even after repeated subcultures. IL-2 and IFN-[gamma] are the two important cytokines responsible for activation and proliferation of CD4 and CD8 T cells. In the

presence of MSCs in the culture, addition of these cytokines fails to cause proliferation of T cells [129]. Some researchers have shown that CD8+ cells are the main target cells that do not proliferate in the presence of MSCs [169]. Emergence of a population of CD8+ regulatory T cells has been suggested to mediate the inhibitory effects of MSCs. Further research is needed to elucidate the mechanism of inhibitory actions of MSCs on T lymphocytes. MSCs inhibit cytotoxic T-lymphocyte mediated lysis in a time and dose-dependent manner [172]. In a 6-day culture, when MSCs are present in adequate number from day 1 (1: 10), they completely prevent lysis of target blast cells. If added on day 3, this effect is mild and if added in small number (1: 1000 ratio) there is no effect at all [172]. Inhibition is marked when a large number of MSCs are present (MSC to lymphocyte ratio of >1: 10). With lower concentrations of MSCs, inhibition is less consistent or can even enhance lymphocyte proliferation (1: 100 to 1: 10 000) [164,174]. Similarly, suppression of proliferation of CD4 and CD8 cells is most pronounced if MSCs are added at the beginning of the culture [117,118].

3. Clinical implications

Bone marrow contains pluripotent cells that can not only differentiate into mesenchymal tissues of multiple lineages, but also modulate immune responses. They do not require MHC expression to exert their inhibitory effect. They can be derived from a donor irrespective of their MHC haplotype and can be kept ready for use in any other patient. Data suggest that immunologic properties of MSCs can be seen both in vitro and in vivo, though in-vivo data are limited.

If these immunomodulatory properties of bone marrow cells can be put into practice, there are several exciting therapeutic options: (1) Treatment of heart failure and ischemic heart disease: allogeneic bone marrow cells can be harvested, expanded in culture, stored and used in acute (following acute myocardial infarction) as well as chronic settings; (2) Bone marrow transplantation: MSCs have a significant therapeutic potential in allogeneic transplantation. They can improve engraftment yield by their antiproliferative effects on T lymphocytes ;(3) Prevention of GVHD [175,176]: GVHD develops because of alloreactive T lymphocytes present in the allograft. MSCs, because of their ability to inhibit IFN-[gamma] secretion and increase IL-4 production, can decrease the overall

incidence of acute and chronic GVHD [189]. Their success in prolonging skin graft survival in the baboon model has prompted clinical trials in acute GVHD with promising results. Le Blanc et al. [175] have shown striking clinical benefit in a patient suffering from GVHD; (4) Modulation of inflammatory response; (5) Prevention of graft rejection; (6) Vehicles for gene therapy.

MSCs are attractive options for both cellular therapy and gene therapy. They are potentially good vehicles for gene therapy as they will not be lost through differentiation as rapidly as haematopoietic progenitors [186]. Allogeneic MSCs can be prepared commercially for use in acute settings. Considering their immense clinical potential, recently efforts have been made to define optimal culture conditions for large scale production of MSCs. bFGF is the most common growth supplement used in MSC culture media [186]. In spite of up-regulation of HLA-I and HLA-DR expression by bFGF, MSCs exhibit enhanced immunosuppressive potential. Use of bFGF, however, favors differentiation towards osteogenic lineage [187]. Further research is needed to enhance clinical scale production of MSCs. Some researchers have drawn attention towards the potential side effect of suppressing patient's antitumour response. MSCs can trigger angiogenesis through secretion of VEGF and support tumour development. We need to probe further to clarify its potential clinical implications.

4. Conclusions

The potential use of MSCs in cellular therapy has prompted researchers to look into their interaction with the host immune response as they have multipotential differentiation abilities and unique immunomodulatory properties. Lots of literature suggests that allogeneic MSCs do not activate T lymphocytes even in the presence of T-cell growth factors [129]. In-vitro data show that MSCs escape recognition by immune system, but limited in-vivo data exist. More research to collect in-vivo data and clinical trials are required to assess and improve the efficacy of MSC treatment for heart failure. Controversies not withstanding, use of MSCs in regenerative medicine is one of the major goals to be achieved by the scientific community in coming years. Owing to their functional characteristics, MSCs are being evaluated for several potential clinical applications like gene therapy, modulation of GVHD, enhance haematopoietic recovery

following myeloablative therapy and improve engraftment following allogeneic bone marrow transplantation [189]. However, the molecular mechanisms mediating the immunosuppressive effects of MSCs remain controversial. Even as we move towards clinical applications of bone marrow-derived MSCs for treatment of heart failure, the exact cellular and molecular mechanisms leading to improvement in ventricular performance also remain controversial. There is a growing perception amongst researchers that the quality and quantity of myocardial regeneration induced by cellular therapy is insufficient to explain the clinical benefits reported. Paracrine actions of MSCs and some as yet undefined mechanisms might have an important role to play.

Further experimental and clinical trials are needed to fully characterize the paracrine and immunomodulatory effects of MSCs if we want to realize the full potential of stem cells in tissue regeneration. We need more information about the pharmacokinetics of transplanted MSCs. What MSCs do to the host tissues and how friendly or hostile the hosts tissues are towards their guests (implanted cells) are the areas where we need to concentrate our future research efforts. Answer to these critical questions will eventually allow large-scale production of allogeneic MSCs and their clinical applications. The future holds much promise for use of allogeneic MSCs.

REFERENCES

1. Merrill CT, Elixhauser A. Hospitalization in the United States, 2002: HCUP Fact Book No. 6. Rockville, MD: Agency for Healthcare Research and Quality; 2005. AHRQ publication 05-0056.
2. Institute of Medicine. Rewarding Provider Performance: Aligning Incentives in Medicare. Washington, DC: National Academy Press; 2007.
3. Centers for Medicare & Medicaid Services Hospital Pay-for-Performance Workgroup. US Dept of Health and Human Services Medicare hospital value-based purchasing plan development: issues paper, 1st public listening session, January17,2007.http://www.cms.hhs.gov AcuteInpatientPPS/Downloads/Hospital_VBP _Plan_Issues_Paper.pdf. Accessed July 17, 2007.
4. Krumholz HM, Parent EM, Tu N, et al. Readmission after hospitalization for congestive heart failure among Medicare beneficiaries. Arch Intern Med. 1997;157(1):99-104.
5. Gwadry-Sridhar FH, Flintoft V, Lee DS, Lee H, Guyatt GH. A systematic review and meta-analysis of studies comparing readmission rates and mortality rates in patients with heart failure. Arch Intern Med. 2004;164(21):2315-2320.
6. Phillips CO, Wright SM, Kern DE, Singa RM, Shepperd S, Rubin HR. Comprehensive discharge planning with postdischarge support for older patients with congestive heart failure: a metaanalysis [published correction appears in JAMA. 2004;292(9):1022]. JAMA. 2004;291(11): 1358-1367.
7. Chiu RCJ, Zibaitis A, Kao RL. Cellular cardiomyoplasty: myocardial regeneration with statellite cell implantation. Ann. Thorac Surg 1995; 60:12-18
8. Friedenstein AJ, Gorskaja U, Kalugina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4: 267-274
9. Prockop DJ. Marrow stromal cells as stem cells for nonhaematopoietic tissue. Science. 1997;276:71-74
10. Wang JS, Shum-Tim D, Chedrawy E, Chiu RC.The coronary delivery of marrow stromal cells for myocardial regeneration: pathophysiologic and therapeutic implications. J Thorac Cardiovasc Surg. 2001 Oct;122(4):699-705.

11. Gurfinkel E; on behalf of the Ushuaia Conference Group. Current Treatment and Future Prospects for the Management of Acute Coronary Syndromes: Consensus Recommendations of the 1997 Ushuaia Conference, Tierra Del Fuego, Argentina. Clin Drug Investig. 1998 May;15(5):367-380.
12. Kereiakes DJ. Inflammation as a therapeutic target: a unique role for abciximab. Am Heart J 2003;146(Suppl. 4):S1–S4.
13. Kereiakes DJ. Adjunctive pharmacotherapy before percutaneous coronary intervention in non-ST-elevation acute coronary syndromes: the role of modulating inflammation. Circulation 2003;108(16 Suppl. 1):III22–III27.
14. Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. Cardiovasc Res 2002;53:31–47.
15. Minatoguchi S, Takemura G, Chen XH et al. Acceleration of the healing process and myocardial regeneration may be important as a mechanism of improvement of cardiac function and remodeling by postinfarction granulocyte colony-stimulating factor treatment. Circulation 2004;109:2572–2580.
16. Barandon L, Couffinhal T, Dufourcq P et al. Frizzled A, a novel angiogenic factor: promises for cardiac repair. Eur J ardiothorac Surg,2004;25:76–83.
17. White HD, Norris RM, Brown MA, Brandt PW, Whitlock RM, and Wild CJ. Left ventricular end-systolic volume as the major determinant of survival after recovery from myocardial infarction. Circulation 76: 44–51, 1987.
18. St John Sutton M, Pfeffer MA, Plappert T, Rouleau JL, Moye LA, Dagenais GR, Lamas GA, Klein M, Sussex B, Goldman S, and et al. Quantitative two-dimensional echocardiographic mea- surements are major predictors of adverse cardiovascular events after acute myocardial infarction: the protective effects of captopril. Circulation 89: 68–75, 1994.
19. Sogaard P, Gotzsche CO, Ravkilde J, Norgaard A, and Thygesen K. Ventricular arrhythmias in the acute and chronic phases after acute myocardial infarction: effect of intervention with captopril. Circulation 90: 101–107, 1994.
20. Maekawa N, Wada H, Kanda T, Niwa T, Yamada Y, Saito K, Fujiwara H, Sekikawa K, and Seishima M. Improved myocardial ischemia/reperfusion injury

in mice lacking tumor necrosis factor-alpha. J Am Coll Cardiol 39: 1229–1235, 2002.

21. Smith, M. A., Court, E. L., and Smith, J. G. (2001) Stem cell factor: laboratory and clinical aspects. Blood Rev. 15, 191– 197
22. Broudy, V. C., Lin, N. L., Priestley, G. V., Nocka, K., and Wolf, N. S. (1996) Interaction of stem cell factor and its receptor c-kit mediates lodgment and acute expansion of hematopoietic cells in the murine spleen. Blood 88, 75–81
23. Heissig, B., Hattori, K., Dias, S., Friedrich, M., Ferris, B., Hackett, N. R., Crystal, R. G., Besmer, P., Lyden, D., Moore, M. A., Werb, Z., and Rafii, S. (2002) Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 109, 625–637
24. Strieter RM, Polverini PJ, Arenberg DA, Walz A, Opdenakker G, Van Damme J, and Kunkel SL. Role of C-X-C chemokines as regulators of angiogenesis in lung cancer. J Leukoc Biol 57: 752–762, 1995.
25. Sun Y and Weber KT. Infarct scar: a dynamic tissue. Cardiovasc Res 46: 250–256, 2000.
26. Stanton LW, Garrard LJ, Damm D, Garrick BL, Lam A, Kapoun AM, Zheng Q, Protter AA, Schreiner GF, and White RT. Altered patterns of gene expression in response to myocardial infarction. Circ Res 86: 939–945, 2000.
27. Stirling GA and Kakkar VV. Cells in the circulating blood capable of producing connective tissue. Br J Exp Pathol 50: 51–55, 1969.
28. Streit M, Velasco P, Brown LF, Skobe M, Richard L, Riccardi L, Lawler J, and Detmar M. Overexpression of thrombospondin-1 decreases angiogenesis and inhibits the growth of human cutaneous squamous cell carcinomas. Am J Pathol 155: 441–452, 1999.
29. Related Articles,Moro C, Jouan MG, Rakotovao A, Toufektsian MC, Ormezzano O, Nagy N, Tosaki A, de Leiris J, Boucher F. Delayed expression of cytokines after reperfused myocardial infarction: possible trigger for cardiac dysfunction and ventricular remodeling. Am J Physiol Heart Circ Physiol. 2007Nov;293(5):H3014-9. Epub 2007 Sep 14.

30. Dewald O, Ren G, Duerr GD, Zoerlein M, Klemm C, Gersch C, Tincey S, Michael LH, Entman ML, and Frangogiannis NG. Of mice and dogs: species-specific differences in the inflammatory response following myocardial infarction. Am J Pathol 164: 665–677, 2004.
31. Dewald O, Zymek P, Winkelmann K, Koerting A, Ren G, Abou- Khamis T, Michael LH, Rollins BJ, Entman ML, and Frangogiannis NG. CCL2/Monocyte chemoattractant protein-1 regulates inflammatory responses critical to healing myocardial infarcts. Circ Res 96: 881–889, 2005.
32. Dimmeler S, Zeiher AM, and Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. J Clin Invest 115: 572–583, 2005.
33. Kishimoto T, Akira S, Narazaki M, and Taga T. Interleukin-6 family of cytokines and gp130. Blood 86: 1243–1254, 1995.
34. Ding ZM, Babensee JE, Simon SI, Lu H, Perrard JL, Bullard DC, Dai XY, Bromley SK, Dustin ML, Entman ML, Smith CW, and Ballantyne CM. Relative contribution of LFA-1 and Mac-1 to neutrophil adhesion and migration. J Immunol 163: 5029– 5038, 1999.
35. Dewald O, Ren G, Duerr GD, Zoerlein M, Klemm C, Gersch C, Tincey S, Michael LH, Entman ML, and Frangogiannis NG. Of mice and dogs: species-specific differences in the inflammatory response following myocardial infarction. Am J Pathol 164: 665–677, 2004.
36. Herskowitz A, Choi S, Ansari AA, and Wesselingh S. Cytokine mRNA expression in postischemic/reperfused myocardium. Am J Pathol 146: 419–428, 1995.
37. Guillen I, Blanes M, Gomez-Lechon MJ, and Castell JV. Cytokine signaling during myocardial infarction: sequential appearance of IL-1 beta and IL-6. Am J Physiol 269: R229–R235, 1995.
38. Hwang MW, Matsumori A, Furukawa Y, Ono K, Okada M, Iwasaki A, Hara M, Miyamoto T, Touma M, and Sasayama S. Neutralization of interleukin-1beta in the acute phase of myocardial infarction promotes the progression of left ventricular remodeling. J Am Coll Cardiol 38: 1546–1553, 2001.

39. Gurantz D, Cowling RT, Varki N, Frikovsky E, Moore CD, and Greenberg BH. IL-1ßeta and TNF-_lpha up-regulate angiotensin II type 1 (AT1) receptors on cardiac fibroblasts and are associated with increased AT1 density in the post-MI heart. J Mol Cell Cardiol 38: 505–515, 2005.
40. Siwik DA, Chang DL, and Colucci WS. Interleukin-1beta and tumor necrosis factor-alpha decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts in vitro. Circ Res 86: 1259–1265, 2000.
41. Aoyama T, Takimoto Y, Pennica D, Inoue R, Shinoda E, Hattori R, Yui Y, and Sasayama S. Augmented expression of cardiotrophin- 1 and its receptor component, gp130, in both left and right ventricles after myocardial infarction in the rat. J Mol Cell Cardiol 32: 1821–1830, 2000.
42. Frangogiannis NG, Lindsey ML, Michael LH, Youker KA, Bressler RB, Mendoza LH, Spengler RN, Smith CW, and Entman ML. Resident cardiac mast cells degranulate and release preformed TNF-_lpha, initiating the cytokine cascade in experimental canine myocardial ischemia/reperfusion. Circulation 98: 699–710, 1998.
43. Gwechenberger M, Mendoza LH, Youker KA, Frangogiannis NG, Smith CW, Michael LH, and Entman ML. Cardiac myocytes produce interleukin-6 in culture and in viable border zone of reperfused infarctions. Circulation 99: 546–551, 1999.
44. Kukielka GL, Smith CW, Manning AM, Youker KA, Michael LH, and Entman ML. Induction of interleukin-6 synthesis in the myocardium: potential role in postreperfusion inflammatory injury. Circulation 92: 1866–1875, 1995.
45. Kereiakes DJ. Inflammation as a therapeutic target: a unique role for abciximab. Am Heart J 2003;146(Suppl. 4):S1–S4.
46. Wollert KC and Drexler H. The role of interleukin-6 in the failing heart. Heart Fail Rev 6: 95–103, 2001.
47. Aoyama T, Takimoto Y, Pennica D, Inoue R, Shinoda E, Hattori R, Yui Y, and Sasayama S. Augmented expression of cardiotrophin- 1 and its receptor component, gp130, in both left and right ventricles after myocardial infarction in the rat. J Mol Cell Cardiol 32: 1821–1830, 2000.

48. Compton SJ, Cairns JA, Holgate ST, and Walls AF. The role of mast cell tryptase in regulating endothelial cell proliferation, cytokine release, and adhesion molecule expression: tryptase induces expression of mRNA for IL-1 beta and IL-8 and stimulates the selective release of IL-8 from human umbilical vein endothelial cells. J Immunol 161: 1939–1946, 1998.

49. Fuchs M, Hilfiker A, Kaminski K, Hilfiker-Kleiner D, Guener Z, Klein G, Podewski E, Schieffer B, Rose-John S, and Drexler H. Role of interleukin-6 for LV remodeling and survival after experimental myocardial infarction. FASEB J 17: 2118–2120, 2003.

50. Eddy LJ, Goeddel DV, Wong GH. Tumor necrosis factor-alpha pretreatment is protective in a rat model of myocardial ischemia-reperfusion injury. Biochem Biophys Res Commun. 1992 Apr 30;184(2):1056-9.

51. Ivey CL, Williams FM, Collins PD, Jose PJ, Williams TJ. Neutrophil chemoattractants generated in two phases during reperfusion of ischemic myocardium in the rabbit: evidence for a role for C5a and interleukin-8. J Clin Invest 1995;95:2720-2728.

52. Kukielka GL, Smith CW, LaRosa GJ, Manning AM, Mendoza LH, Daly TJ, Hughes BJ, Youker KA, Hawkins HK, Michael LH, Rot A, Entman ML. Interleukin-8 gene induction in the myocardium after ischemia and reperfusion in vivo. J Clin Invest 1995;95:89-103.

53. Wan S, LeClerc JL, Vincent JL. Cytokine responses to cardiopulmonary bypass: lessons learned from cardiac transplantation. Ann Thorac Surg 1997;63:269-276.

54. Wan S, DeSmet JM, Barvais L, Goldstein M, Vincent JL, LeClerc JL. Myocardium is a major source of proin¯ammatory cytokines in patients undergoing cardiopulmonary bypass. J Thorac Cardiovasc Surg 1996;112:806-811.

55. Riesenberg K, Levy R, Katz A, Galkop S, Schlaeffer F. Neutrophil superoxide release and interleukin 8 in acute myocardial infarction: distinction between complicated and uncomplicated states. Eur J Clin Invest 1997;27:398-404.

56. Kilgore KS, Park JL, Tanhehco EJ, Booth EA, Marks RM, Lucchesi BR. Attenuation of interleukin-8 expression in C6-de®cient rabbits after myocardial ischemia/reperfusion. J Mol Cell Cardiol 1998;30:75-85.
57. Boyle Jr EM, Kovacich JC, HeÁbert CA, Canty TG, Chi E, Morgan EN, Pohlman TH, Verrier ED. Inhibition of interleukin-8 blocks myocardial ischemia-reperfusion injury. J Thorac Cardiovasc Surg 1998;116:114-121.
58. Sekido N, Mukaida N, Harada A, Nakanishi I, Watanabe Y, Matsushima K. Prevention of lung reperfusion injury in rabbits by a monoclonal antibody against interleukin-8. Nature 1993;365:654-657.
59. Matsumoto T, Yokoi K, Mukaida N, Harada A, Yamashita J, Watanabe Y, Matsushima K. Pivotal role of interleukin-8 in the acute respiratory distress syndrome and cerebral reperfusion injury. J Leukoc Biol 1997;62:581-587.
60. Wan S, Izzat MB, Lee TW, Wan IYP, Tang NLS, Yim APC. Avoiding cardiopulmonary bypass in multivessel-CABG reduces cytokine response and myocardial injury. Ann Thorac Surg 1999;68:52-57.
61. Jimenez MF, Watson RW, Parodo J, Evans D, Foster D, Steinberg M, Rotstein OD, Marshall JC. Dysregulated expression of neutrophil apoptosis in the systemic in-inflammatory response syndrome. Arch Surg 1997;132:1263-1270.
62. Goodman ER, Kleinstein E, Fusco AM, Quinlan DP, Lavery R, Livingston DH, Deitch EA, Hauser CJ. Role of interleukin 8 in the genesis of acute respiratory distress syndrome through an effect on neutrophil apoptosis. Arch Surg 1998;133:1234-1239.
63. Simonini A, Moscucci M, Muller DW, Bates ER, Pagani FD, Burdick MD, Strieter RM. IL-8 is an angiogenic factor in human coronary atherectomy tissue. Circulation. 2000 Apr 4;101(13):1519-26
64. S . Vandervelde , M . van Luyn , M . Rozenbaum , A . Petersen , R . Tio , M . Harmsen. Stem cell-related cardiac gene expression early after murine myocardial infarction . Cardiovascular Research , Volume 73 , Issue 4 , Pages 783 - 793
65. Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, and Mosmann TR. Interleukin-10. Annu Rev Immunol 11: 165–190, 1993.

66. Mosmann TR. Properties and functions of interleukin-10. Adv Immunol 56: 1–26, 1994.

67. Yang Z, Zingarelli B, and Szabo C. Crucial role of endogenous interleukin-10 production in myocardial schemia/reperfusion injury. Circulation 101: 1019–1026, 2000.

68. Frangogiannis NG, Mendoza LH, Lindsey ML, Ballantyne CM, Michael LH, Smith CW, and Entman ML. IL-10 is induced in the reperfused myocardium and may modulate the reaction to injury. J Immunol 165: 2798–2808, 2000.

69. Lacraz S, Nicod LP, Chicheportiche R, Welgus HG, and Dayer JM. IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. J Clin Invest 96: 2304–2310, 1995.

70. Shahzad G Raja, Gilles D Dreyfus. Modulation of Systemic Inflammatory Response after Cardiac Surgery. *Asian Cardiovasc Thorac Ann 2005; 13:382-395*

71. Richard I. Hall, Mark Stafford Smith and Graeme Rocker. The Systemic Inflammatory Response to Cardiopulmonary Bypass: Pathophysiological, Therapeutic, and Pharmacological Considerations. Anesth Analg 1997; 85:766-82

72. Nesher N, Frolkis I, Vardi M, Sheinberg N, Bakir I, Caselman F, Pevni D, Ben-Gal Y, Sharony R, Bolotin G, Loberman D, Uretzky G, Weinbroum AA. Higher levels of serum cytokines and myocardial tissue markers during on-pump versus off-pump coronary artery bypass surgery. J Card Surg. 2006 Jul-Aug;21(4):395-402

73. Pasqui AL, Di Renzo M, Bova G, Maffei S, Pompella G, Auteri A, Puccetti L. Pro-inflammatory/anti-inflammatory cytokine imbalance in acute coronary syndromes. Clin Exp Med. 2006 Mar; 6(1): 38-44.

74. T Kilic, D Ural, E Ural, Z Yumuk, An Agacdiken, T Sahin, G Kahraman, G Kozdag, A Vural, B Komsuoglu. Relation between proinflammatory to anti-inflammatory cytokine ratios and long-term prognosis in patients with non-ST elevation acute coronary syndrome. Heart 2006;92:1041–1046.

75. Mazzone A, De Servi S, Vezzoli M, Fossati G, Mazzucchelli I, Gritti D, Ottini E, Mussini A, Specchia G (1999) Plasma levels of interleukin-2, -6,-10 and

phenotypic characterization of circulating T-lymphocytes in ischemic heart disease. Atherosclerosis 145:369–734

76. Lu L, Zhang JQ, Ramires FJ, Sun Y. Molecular and cellular events at the site of myocardial infarction: from the perspective of rebuilding myocardial tissue. Biochem Biophys Res Commun. 2004 Jul 30;320(3):907-13.
77. Moro C, Jouan MG, Rakotovao A, Toufektsian MC, Ormezzano O, Nagy N, Tosaki A, de Leiris J, Boucher F. Delayed expression of cytokines after reperfused myocardial infarction: possible trigger for cardiac dysfunction and ventricular remodeling. Am J Physiol Heart Circ Physiol. 2007 Nov;293(5):H3014-9. Epub 2007 Sep 14.
78. Litt MR, Jeremy RW, Weisman HF et al. Neutrophil depletion limited to reperfusion reduces myocardial infarct size after 90 minutes of ischemia. Evidence for neutrophil-mediated reperfusion injury. Circulation 1989;80:1816–1827.
79. Engler RL. Free radical and granulocyte-mediated injury during myocardial ischemia and reperfusion. Am J Cardiol 1989;63:19E–23E.
80. Kereiakes DJ. Inflammation as a therapeutic target: a unique role for abciximab. Am Heart J 2003;146(Suppl. 4):S1–S4.
81. Wan S, LeClerc JL, Vincent JL. Inflammatory response to cardiopulmonary bypass: mechanisms involved and possible therapeutic strategies. Chest 1997;112:676–692
82. Danenberg HD, Fishbein I, Gao J et al. Macrophage depletion by clodronate-containing liposomes reduces neointimal formation after balloon injury in rats and rabbits. Circulation 2002;106:599–605.
83. Serini G, Gabbiani G. Mechanisms of myofibroblast activity and phenotypic modulation. Exp Cell Res 1999;250:273–283.
84. Minatoguchi S, Takemura G, Chen XH et al. Acceleration of the healing process and myocardial regeneration may be important as a mechanism of improvement of cardiac function and remodeling by postinfarction granulocyte colony-stimulating factor treatment. Circulation 2004;109:2572–2580.

85. Flaherty JT, Pitt B, Gruber JW, Heuser RR, Rothbaum DA, Burwell LR, George BS, Kereiakes DJ, Deitchman D, Gustafson N, and et al. Recombinant human superoxide dismutase (h-SOD) fails to improve recovery of ventricular function in patients undergoing coronary angioplasty for acute myocardial infarction. *Circulation* 89: 1982–1991, 1994.
86. Nathan C. Points of control in inflammation. *Nature* 420: 846–852, 2002.
87. Haslett C. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *Am J Respir Crit Care Med* 160: S5–S11, 1999.
88. Chilvers ER, Cadwallader KA, Reed BJ, White JF, and Condliffe AM. The function and fate of neutrophils at the inflamed site: prospects for therapeutic intervention. *J R Coll Physicians* Lond 34: 68–74, 2000.
89. Hart SP, Dougherty GJ, Haslett C, and Dransfield I. CD44 regulates phagocytosis of apoptotic neutrophil granulocytes, but not apoptotic lymphocytes, by human macrophages. *J Immunol* 159: 919–925, 1997.
90. Huynh ML, Fadok VA, and Henson PM. Phosphatidylserinedependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *J Clin Invest* 109: 41–50, 2002.
91. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, and Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest* 101: 890–898, 1998.
92. Creemers EE, Cleutjens JP, Smits JF, and Daemen MJ. Matrix metalloproteinase inhibition after myocardial infarction: a new approach to prevent heart failure? *Circ Res* 89: 201–210, 2001.
93. Nagase H, Visse R, and Murphy G. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res* 69: 562–573, 2006.
94. Montfort I and Perez-Tamayo R. The distribution of collagenase in normal rat tissues. *J Histochem Cytochem* 23: 910–920, 1975.
95. Cleutjens JP, Kandala JC, Guarda E, Guntaka RV, and Weber KT. Regulation of collagen degradation in the rat myocardium after infarction. *J Mol Cell Cardiol* 27: 1281–1292, 1995.

96. Siwik DA, Chang DL, and Colucci WS. Interleukin-1beta and tumor necrosis factor-alpha decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts in vitro. *Circ Res* 86: 1259–1265, 2000.

97. Laiho M, Saksela O, Andreasen PA, and Keski-Oja J. Enhanced production and extracellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-beta. *J Cell Biol* 103: 2403–2410, 1986.

98. Verrecchia F, Chu ML, and Mauviel A. Identification of novel TGF-beta/Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. *J Biol Chem* 276: 17058–17062, 2001.

99. Gearing AJ, Beckett P, Christodoulou M, Churchill M, Clements J, Davidson AH, Drummond AH, Galloway WA, Gilbert R, Gordon JL, and et al. Processing of tumour necrosis factor-alpha precursor by metalloproteinases. Nature 370: 555–557, 1994

100. McQuibban GA, Gong JH, Wong JP, Wallace JL, Clark-Lewis I, and Overall CM. Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. Blood 100: 1160–1167, 2002.

101. Rohde LE, Ducharme A, Arroyo LH, Aikawa M, Sukhova GH, Lopez-Anaya A, McClure KF, Mitchell PG, Libby P, and Lee RT. Matrix metalloproteinase inhibition attenuates early left ventricular enlargement after experimental myocardial infarction in mice. *Circulation* 99: 3063–3070, 1999.

102. Lindsey ML, Mann DL, Entman ML, and Spinale FG. Extracellular matrix remodeling following myocardial injury. *Ann Med* 35: 316–326, 2003.

103. Vanhoutte D, Schellings M, Pinto Y, and Heymans S. Relevance of matrix metalloproteinases and their inhibitors after myocardial infarction: a temporal and spatial window. *Cardiovasc Res* 69: 604–613, 2006.

104. Villarreal FJ, Griffin M, Omens J, Dillmann W, Nguyen J, and Covell J. Early short-term treatment with doxycycline modulates postinfarction left ventricular remodeling. *Circulation* 108: 1487–1492, 2003.

105. Heymans S, Luttun A, Nuyens D, Theilmeier G, Creemers E, Moons L, Dyspersin GD, Cleutjens JP, Shipley M, Angellilo A, Levi M, Nube O, Baker A, Keshet E, Lupu F, Herbert JM, Smits JF, Shapiro SD, Baes M, Borgers M, Collen D, Daemen MJ, and Carmeliet P. Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. *Nat Med* 5: 1135–1142,1999.

106. Ducharme A, Frantz S, Aikawa M, Rabkin E, Lindsey M, Rohde LE, Schoen FJ, Kelly RA, Werb Z, Libby P, and Lee RT. Targeted deletion of matrix metalloproteinase-9 attenuates left ventricular enlargement and collagen accumulation after experimental myocardial infarction. *J Clin Invest* 106: 55–62, 2000.

107. Hayashidani S, Tsutsui H, Ikeuchi M, Shiomi T, Matsusaka H, Kubota T, Imanaka-Yoshida K, Itoh T, and Takeshita A. Targeted deletion of MMP-2 attenuates early LV rupture and late remodeling after experimental myocardial infarction. *Am J Physiol Heart Circ Physiol* 285: H1229–H1235, 2003

108. Matsumura S, Iwanaga S, Mochizuki S, Okamoto H, Ogawa S, and Okada Y. Targeted deletion or pharmacological inhibition of MMP-2 prevents cardiac rupture after myocardial infarction in mice. J Clin Invest 115: 599–609, 2005.

109. Fang KC, Wolters PJ, Steinhoff M, Bidgol A, Blount JL, and Caughey GH. Mast cell expression of gelatinases A and B is regulated by kit ligand and TGF-beta. J Immunol 162: 5528–5535, 1999.

110. Roten L, Nemoto S, Simsic J, Coker ML, Rao V, Baicu S, Defreyte G, Soloway PJ, Zile MR, and Spinale FG. Effects of gene deletion of the tissue inhibitor of the matrix metalloproteinasetype 1 (TIMP-1) on left ventricular geometry and function in mice. J Mol Cell Cardiol 32: 109–120, 2000.

111. Creemers EE, Davis JN, Parkhurst AM, Leenders P, Dowdy KB, Hapke E, Hauet AM, Escobar PG, Cleutjens JP, Smits JF, Daemen MJ, Zile MR, and Spinale FG. Deficiency of tissue inhibitor of matrix metalloproteinase-1 exacerbates LV remodeling following myocardial infarction in mice. 2003 Jan;284(1):H364-71. Epub 2002 Sep 26

112. Ikonomidis JS, Hendrick JW, Parkhurst AM, Herron AR, Escobar PG, Dowdy KB, Stroud RE, Hapke E, Zile MR, and Spinale FG. Accelerated LV remodeling after myocardial infarction in TIMP-1-deficient mice: effects of exogenous MMP inhibition. Am J Physiol Heart Circ Physiol 288: H149–H158, 2005.

113. Heymans S, Luttun A, Nuyens D, Theilmeier G, Creemers E, Moons L, Dyspersin GD, Cleutjens JP, Shipley M, Angellilo A, Levi M, Nube O, Baker A, Keshet E, Lupu F, Herbert JM, Smits JF, Shapiro SD, Baes M, Borgers M, Collen D, Daemen MJ, and Carmeliet P. Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. Nat Med 5: 1135–1142, 1999.

114. Jaquet K, Krause KT, Denschel J, Faessler P, Nauerz M, Geidel S, et al. Reduction of myocardial scar size after implantation of mesenchymal stem cells in rats: what is the mechanism? Stem Cells Dev 2005; 14:299–309

115. Menasche P. Cellular transplantation: hurdles remaining before widespread clinical use. Curr Opin Cardiol 2004; 19:154–161

116. Pfeffer MA. Left ventricular remodeling after acute myocardial infarction. Annu Rev Med 1995; 46:455–466.

117. Chachques JC, Acar C, Herreros J, Trainini JC, Prosper F, D'Attellis N, et al. Cellular cardiomyoplasty: clinical application. Ann Thorac Surg 2004; 77:1121–1130

118. Nishida M, Li TS, Hirata K, Yano M, Matsuzaki M, Hamano K. Improvement of cardiac function by bone marrow cell implantation in a rat hypoperfusion heart model. Ann Thorac Surg 2003; 75:768–774.

119. Xu X, Xu Z, Xu Y, Cui G. Effects of mesenchymal stem cell transplantation on extracellular matrix after myocardial infarction in rats. Coron Artery Dis 2005; 16:245–255.

120. Orlic D, Hill JM, Arai AE. Stem cells for myocardial regeneration. Circ Res 2002; 91:1092–1102.

121. Siepe M, Heilmann, von Samson P, Menasche P, Beyersdorf F. Stem cell research and cell transplantation for myocardial regeneration. Eur J Cardiothorac Surg 2005; 28:318–324.

122. Pagani FD, DerSimonian H, Zawadzka A, Wetzel K, Edge AS, Jacoby DB, et al. Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation. J Am Coll Cardiol 2003; 41:879–888.

123. Li RK, Jia ZQ, Weisel RD, Mickle DA, Zhang J, Mohabeer MK, et al. Cardiomyocyte transplantation improves heart function. Ann Thorac Surg 1996; 62:654–660.

124. Sakai T, Li RK, Weisel RD, Mickle DA, Kim EJ, Tomita S, et al. Autologous heart cell transplantation improves cardiac function after myocardial injury. Ann Thorac Surg 1999; 68:2074–2081.

125. Gronthos S, Simmons PJ. The biology and application of human bone marrow stromal cell precursors. J Hematother 1996; 5:15–23

126. Friedenstein AJ, Petrokova KV. Osteogenesis in transplants of bone marrow cells. Journal of Embryological Experimental Morphology 1966; 16:381–390.

127. Haynesworth SE, Goshima J, Goldberg VM, Caplan AI. Characterization of cells with osteogenic potential from human marrow. Bone 1992; 13:81– 88.

128. Barry FP. Biology and clinical applications of mesenchymal stem cells. Birth Defects Res C Embryo Today 2003; 69:250–256.

129. Plumas J, Chaperot L, Richard MJ, Molens JP, Bensa JC, Favrot MC. Mesenchymal stem cells induce apoptosis of activated T cells. Leukemia 2005; 19:1597–1604

130. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284:143–147.

131. Jorgensen C, Djouad F, Apparailly F, Noel D. Engineering mesenchymal stem cells for immunotherapy. Gene Ther 2003; 10:928–931

132. Barry FP. Biology and clinical applications of mesenchymal stem cells. Birth Defects Res C Embryo Today 2003; 69:250–256

133. Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol 2004; 36: 568–584.

134. Le Blanc K, Ringden O. Immunobiology of human mesenchymal stem cells and future use in hematopoietic stem cell transplantation. Biol Blood Marrow Transplant 2005; 11:321–334.
135. Le Blanc K, Pittenger M. Mesenchymal stem cells: progress toward promise. Cytotherapy 2005; 7:36–45.
136. Le Blanc K. Immunomodulatory effects of fetal and adult mesenchymal stem cells. Cytotherapy 2003; 5:485–489.
137. El-Badri NS, Maheshwari A, Sanberg PR. Mesenchymal stem cells in autoimmune disease. Stem Cells Dev 2004; 13:463–472
138. Ryan JM, Barry FP, Murphy JM, Mahon BP. Mesenchymal stem cells avoid allogeneic rejection. J Inflamm (Lond) 2005; 2:8.
139. Pfeffer MA. Left ventricular remodeling after acute myocardial infarction. Annu Rev Med 1995; 46:455–466.
140. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001; 410:701– 705.
141. Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P. Transplanted adult bone marrow cells repair myocardial infarcts in mice. Ann N Y Acad Sci 2001; 938:221–229.
142. Sakai T, Li RK, Weisel RD, Mickle DA, Jia ZQ, Tomita S, et al. Fetal cell transplantation: a comparison of three cell types. J Thorac Cardiovasc Surg 1999; 118:715–724.
143. Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, et al. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet 2003; 361:45–46.
144. Galinanes M, Loubani M, Davies J, Chin D, Pasi J, Bell PR. Autotransplantation of unmanipulated bone marrow into scarred myocardium is safe and enhances cardiac function in humans. Cell Transplant 2004; 13:7–13.
145. Schachinger V, Assmus B, Britten MB, Honold J, Lehmann R, Teupe C, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final one-year results of the TOPCARE-AMI Trial. J Am Coll Cardiol 2004; 44:1690–1699.

146. Rafii S, Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat Med 2003; 9:702–712.

147. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky `MW, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001; 107:1395–1402.

148. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, et al. Neovascularization of ischemic myocardium by human bone-marrowderived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 2001; 7: 430–436.

149. Edelberg JM, Tang L, Hattori K, Lyden D, Rafii S. Young adult bone marrowderived endothelial precursor cells restore aging-impaired cardiac angiogenic function. Circ Res 2002; 90:E89–E93.

150. Yeh ET, Zhang S, Wu HD, Korbling M, Willerson JT, Estrov Z. Transdifferentiation of human peripheral blood CD34þ-enriched cell population into cardiomyocytes, endothelial cells, and smooth muscle cells in vivo. Circulation 2003; 108:2070–2073.

151. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 2004; 428:664–668.

152. Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 2004; 428:668–6.

153. Shake JG, Gruber PJ, Baumgartner WA, Senechal G, Meyers J, Redmond JM, et al. Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. Ann Thorac Surg 2002; 73:1919–1925.

154. Tomita S, Li RK, Weisel RD, Mickle DA, Kim EJ, Sakai T, et al. Autologous transplantation of bone marrow cells improves damaged heart function. Circulation 1999; 100 (Suppl):II247–II256.

155. Wang JS, Shum-Tim D, Chedrawy E, Chiu RC. The coronary delivery of marrow stromal cells for myocardial regeneration: pathophysiologic and therapeutic implications. J Thorac Cardiovasc Surg 2001; 122: 699–705.

156. Tang YL, Zhao Q, Qin X, Shen L, Cheng L, Ge J, et al. Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. Ann Thorac Surg 2005; 80:229–237.

157. Tang YL, Qian K, Zhang YC, Shen L, Phillips MI. Mobilizing of haematopoietic stem cells to ischemic myocardium by plasmid mediated stromal-cell-derived factor-1alpha (SDF-1alpha) treatment. Regul Pept 2005; 125:1–8.

158. Rafii S, Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat Med 2003; 9:702–712.

159. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation 2001; 104:1046–1052.

160. Besse A, Trimoreau F, Praloran V, Denizot Y. Effect of cytokines and growth factors on the macrophage colony-stimulating factor secretion by human bone marrow stromal cells. Cytokine 2000; 12:522–525.

161. Nishida M, Li TS, Hirata K, Yano M, Matsuzaki M, Hamano K. Improvement of cardiac function by bone marrow cell implantation in a rat hypoperfusion heart model. Ann Thorac Surg 2003; 75:768–774.

162. Bick-Forrester J, Lee MS, Makkar RR, Forrester JS. Partial restoration of myocardial function and perfusion by cell therapy following myocardial infarction. Curr Opin Cardiol 2004; 19:631–637.

163. McIntosh K, Bartholomew A. Stromal cell modulation of the immune system. A potential role for mesenchymal stem cells. Graft 2000; 3: 324–328.

164. Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol 2003; 57:11–20

165. Kofidis T, deBruin JL, Tanaka M, Zwierzchoniewska M, Weissman I, Fedoseyeva E, et al. They are not stealthy in the heart: embryonic stem cells trigger cell

infiltration, humoral and T-lymphocyte-based host immune response. Eur J Cardiothorac Surg 2005; 28:461–466.

166. Majumdar MK, Keane-Moore M, Buyaner D, Hardy WB, Moorman MA, McIntosh KR, et al. Characterization and functionality of cell surface molecules on human mesenchymal stem cells. J Biomed Sci 2003; 10:228–241.
167. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 2002; 99:3838–3843.
168. Tse WT, Pendleton JD, Beyer WM, Egalka MC,Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. Transplantation 2003; 75:389–397.
169. Djouad F, Plence P, Bony C, Tropel P, Apparailly F, Sany J, et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 2003; 102:3837–3844.
170. Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol 2002; 30:42–48.
171. Rasmusson I, Ringden O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms. Exp Cell Res 2005; 305:33–41.
172. Rasmusson I, Ringden O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. Transplantation 2003; 76:1208–1213.
173. Potian JA, Aviv H, Ponzio NM, Harrison JS, Rameshwar P. Veto-like activity of mesenchymal stem cells: functional discrimination between cellular responses to alloantigens and recall antigens. J Immunol 2003; 171:3426–3434.
174. Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood 2003; 101:3722– 3729.

175. Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 2004; 363:1439–1441.

176. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005; 105:1815–1822. 66 Van den Eynde B, Gaugler B, van der Bruggen P, Coulie P, Brichard V, Boon T. Human tumour antigens recognized by T-cells: perspectives for new cancer vaccines. Biochem Soc Trans 1995; 23:681–686.

177. Strand S, Galle PR. Immune evasion by tumours: involvement of the CD95 (APO-1/Fas) system and its clinical implications. Mol Med Today 1998; 4:63–68.

178. Mitra R, Singh S, Khar A. Antitumour immune responses. Expert Rev Mol Med 2003; 2003:1–22.

179. Puissant B, Barreau C, Bourin P, Clavel C, Corre J, Bousquet C, et al. Immunomodulatory effect of human adipose tissue- derived adult stem cells: comparison with bone marrow mesenchymal stem cells. Br J Haematol 2005; 129:118–129.

180. Maitra B, Szekely E, Gjini K, Laughlin MJ, Dennis J, Haynesworth SE, et al. Human mesenchymal stem cells support unrelated donor hematopoietic stem cells and suppress T-cell activation. Bone Marrow Transplant 2004; 33:597–604.

181. Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood 2004; 103:4619–4621.

182. Zhang W, Ge W, Li C, You S, Liao L, Han Q, et al. Effects of mesenchymal stem cells on differentiation, maturation, and function of human monocytederived dendritic cells. Stem Cells Dev 2004; 13:263–271.

183. Friedenstein AJ, Petrokova KV. Osteogenesis in transplants of bone marrow cells. Journal of Embryological Experimental Morphology 1966; 16:381–390.

184. Jiang XX, Zhang Y, Liu B, Zhang SX, Wu Y, Yu XD, et al. Human mesenchymal stem cells inhibit differentiation and function of monocytederived dendritic cells. Blood 2005; 105:4120–4126.

185. Klyushnenkova E, Mosca JD, Zernetkina V, Majumdar MK, Beggs KJ, Simonetti DW, et al. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. J Biomed Sci 2005; 12:47–57.

186. Bordignon C, Carlo-Stella C, Colombo MP, De Vincentiis A, Lanata L, Lemoli RM, et al. Cell therapy: achievements and perspectives. Haematologica 1999; 84:1110–1149.

187. Sotiropoulou PA, Perez SA, Salagianni M, Baxevanis CN, Papamichail M. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. Stem Cells 2006; 24: 462–471.

188. Kadri T, Lataillade JJ, Doucet C, Marie A, Ernou I, Bourin P, et al. Proteomic study of Galectin-1 expression in human mesenchymal stem cells. Stem Cells Dev 2005; 14:204–212.

189. Frassoni F, Labopin M, Bacigalupo A, Gluckman E, Rocha V, Bruno B, et al. Expanded mesenchymal stem cells (MSC), co-infused with HLA identical hematopoietic stem cell transplants, reduce acute and chronic graft versus host disease: a matched pair analysis [abstract]. Bone Marrow Transplant 2002; 29 (Suppl 2):S2.

190. Nikolaos g. Frangogiannis. The Mechanistic Basis of Infarct Healing. Antioxidants & Redox Signaling Volume 8, Numbers 11 & 12, 2006

Chapter II

MATERIAL & METHODS

1. Study Rationale:

Background information

Coronary artery disease accounts for 50% of all cardiovascular deaths and remains a major cause of morbidity and mortality. Current therapies for congestive heart failure are associated with limited success, inherent complications and significant cost without addressing the fundamental pathophysiological problem related to the loss of functioning contractile cardiomyocytes [1, 2, and 3]. Therefore, there is a need to look for new therapies.

Recently, animal studies and human clinical trials have shown that bone marrow derived mesenchymal stem cells (MSCs) implantations have significant clinical benefits in patients with myocardial infarction (MI) and CHF [4, 5, 6, 7, and 8]. We and others have reported that autologous MSCs, when transplanted into infracted myocardium, can differentiate into cells of various phenotypes and improve ventricular function. The observed beneficial effects of cell trandplantation have then led to many human clinical trials.

However, to date the mechanisms of cardiac function improvement remains unclear. The possible mechanisms include stem cell transdifferention into cardiomyocytes or cell fusion between the transplanted stem cells and residual cardiomyocytes. It has also been suggested that the MSCs could improve the cardiac function through a paracrine or immunomodulation effect, such as angiogenesis [9,10], different inflammatory cytokines secretion as well as Extra-Cellular Matrix (ECM) deposition [11,12], which affect the remodeling process after MI by changing the balance of MMP2 and TIMP1 [13,14].

In a clinical study, the ratio of the pro-and anti-inflammatory cytokines has been shown to be associated with different prognosis and clinically outcome [15]. Some studies suggested the anti-inflammatory roles of MSCs in early renal functional improvement following acute renal failure [16]. Based on these data, we wish to explore in further details the cytokines profile, and changes in the pro-/anti- inflammatory cytokine ratio in the microenvironment to improve cardiac function following acute coronary occlusion. If successful, this project will provide better insights of the beneficial roles of stem cell

therapy in heart failure and alternate explanation on how MSCs improves cardiac function following MI.

Objectives of this research project

In the current research, we propose to examine the mechanism of functional improvement after MSCs therapy by investigating the cytokines profiles in myocardial infarcted areas with or without stem cell therapy.

2. Animal

Immunocompetent female and male syngeneic Lewis rats (200 to 250 g, Charles River, Quebec, Canada) were used in this study. All procedures were in compliance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996) and the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.

3. Experimental Design

A total of 88 female rats underwent proximal left coronary artery ligations and were randomly assigned to three groups. In Group L (n=25) the rats underwent acute left coronary ligation, and then the left ventricle was punctured at three different sites (Apex, right edge and left edge of the LV) using a 28-gauge syringe, with nothing injected. In Group MI, (n=31), 150uL of Dulbecco's Modified Eagle's Medium (DEME) were directly injected into three different sites around the peri-ischemic area of rat myocardium 5 minutes after coronary ligation. In Group C (n=32), isogeneic male MSCs (3 x106) suspended in Dulbecco's Modified Eagle's Medium were similarly injected after ligation. No immunosuppression was given at any time.

4. Isolation, Culture, and Labeling of Rat MSCs

Rat MSC cultures were prepared according to Caplan's [20]. Briefly, after an overdose of pentobarbital (100mg/Kg) given intraperitoneally, bone marrow stromal cells were harvested by flushing the femurs and tibias with Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100U/ml penicillin

G, 100ug/mg streptomycin, 0.25 ug amphotericin B; All obtained from Gibco Laboratories, Boston, MA) and grown in a humidified atmosphere of 5% CO_2.

Whole marrow was plated in tissue culture dishes, medium was replaced every 3 days and the nonadherent cells were discarded. Each primary culture was passaged twice to 3 new plates, and the cell density of the colonies was grown to approximately 90% confluence. To prevent the MSCs from differentiating or slowing their rate of division, each primary culture was replated to 3 new plates when the cell density within colonies became 80% to 90% confluent, approximately 2 weeks after seeding.

Once these cells were nearly confluent, MSCs were transfected as described previously with pMFG-lac Z plasmid containing ß-galactosidase gene for identification of the transplanted cells in the myocardial scar tissue. The resulting MSCs expressing lacZ were expanded for 4 weeks before transplantation. The cells were allowed to proliferate until at least a 70% confluence was achieved before using them for transduction, the MSCs were trypsinized with 0.05% Trypsin + 0.53 mM EDTA (Gibco Labs) and replated. The next day, these cells were transfected with lac Z retroviral particles from the supernatant of lacZ–GP +AM 12 cells, plus 4µg/mL polybrene for 16 hours. After each transfection, the supernatant is removed and MSCs are kept in complete DMEM medium. In order to have high transfection ratio, we did 3-4 consecutive experiments.

5. X-gal staining for Detection of ß-galactosidase Activity

Seventy-two hours after the last transfection, MSCs were trypsinized and part of the cells was plated in a 35-mm dish for histochemical staining for ß-galactosidase activity, in order to determine the percentage of cells expressing ß-galactosidase. The medium was aspirated from the plates and the cells rinsed with PBS. The cells were fixed at 4 °C in fix solution (2% formaldehyde and 0.2% glutaraldehyde in PBS) for 15 minutes and rerinsed with PBS. Staining for ß-gal was performed with a solution containing 1 mg/mL 5-bromo-4-chloro-3-indoyl- ß-D-galactoside (Xgal), 1 mmol/L ethyleneglycol-bis (ß-aminoethyl-ether)-N, N'-teraacetic acid, 5 mmol/L $K_3Fe(CN)_6O.3H_2O$, 2 mmol/L magnesium chloride, and 0.01% sodium deoxycholate was added. The cells were then incubated at 37°C and protected from light for 16 hours. The presence of blue-labeled cells was then confirmed under light microscopy.

6. Preparation of Cells for Injection

Cells isolated from the rat bone marrow were cultured in complete medium in tissue culture dishes. After labeling, the medium was aspirated and the cells were washed with 6 mL of Hank's balanced salt solution (HBSS). The HBSS was aspirated and 2 mL of trypsin-EDTA was added to detach the cells from the bottom of the dish. The detached cell suspension was mixed with 2 mL of complete medium and placed in a hemocytometer for counting. A volume consisting of $3x10^6$ cells was then collected and centrifuged at 2000rpm for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 15uL to complete medium.

7. Creation of Myocardial Infarction and Intramyocardial Transplantation of MSCs

Female rats were anesthetized with 5% isoflurane (MTC Pharmaceuticals, Cambridge, Ontario, Canada). Animals were intubated with an 18-gauge intravenous catheter and connected to a Harvard rodent ventilator (Harvard Apparatus Co. Inc, Boston, MA) at 85 breaths/minute. Anesthesia was maintained with 3% isoflurane and oxygen at 5-6L/min.

A 1.5-cm left anterolateral thoractomy was performed in the fifth intercostal space under sterile conditions to expose the heart, and the left coronary artery was ligated 1 to 2 mm from its origin with a 7-0 polypropylene non-absorbable suture (Ethicon, Inc, Somerville, NJ). Successful performance of coronary occlusion was verified by the development of a pale color in the ventricle after ligation. After 2-3 minutes, under direct vision, lac-z labeled MSCs ($3x10^6$ cells suspended in 150 uL DMEM) were injected in Group C rats at three different sites (Apex, right edge and left edge of the infarcted area) into the peri-infarcted area of the left ventricle using a 28-gauge syringe. Small blebs under the injected area were confirmed in every case. An equal volume of cell-free 150uL DMEM was injected into Group M. After achieving hemostasis, the muscle layers and skin were closed separately with 4-0 monofilament sutures. Once spontaneous respiration resumed, the experimental animals were extubated and placed in a temperature-controlled chamber until they resumed full alertness and mobility. Furthermore, Buprenorphine hydrochloride (0.01 to 0.05 mg/kg subcutaneously) was given postoperatively for pain control.

8. Cardiac Functional Assessment

Transthoracic echocardiography was performed on all surviving animals in Group L (n = 25), Group M (n =31), and Group C (n =32) before operation (baseline), at 12 and 24 hours, 1 and 2 weeks after ligation and treatment. Echocardiograms were obtained with a commercially available system (SonoSite, Titan-Washington, Seattle, WA) equipped with a 15-MHz transducer. After sedating the animals with 2% isoflurane, echocardiography was performed as described elsewhere [20]. Briefly, parasternal long- and short-axis views were obtained with both M-mode and two-dimensional images. End-diastolic (LVEDD) and end-systolic (LVESD) diameters were measured with M-mode tracings between the anterior and posterior walls from the short-axis view just below the level of the papillary muscles of the mitral valve. Two images on average were obtained in each view and averaged over three consecutive cardiac cycles. This was done according to the American Society of Echocardiology leading-edge method. Fractional shortening (FS) was determined as [(LVEDD-LVESD) / LVEDD] x 100 (%). Left ventricular end-diastolic volume (LVEDV) was calculated as 7.0 x $LVEDD^3$ / (2.4 +LVEDD), and left ventricular end-systolic volume (LVESV) as 7.0 x $LVESD^3$ / (2.4 + LVESD. The ejection fraction (EF) was estimated as (LVEDV- LVESV) / LVEDV. All measurements were performed by one experienced observer (Dr. J.F.A.), who was blinded to the treatment groups.

9. Hearts Harvest and Tissue Processing

Animal were sacrificed at 12 and 24 hours, 1 and 2 weeks after the operation and echicardiograph, the hearts of animals were harvested, quickly dissected out, and the whole left ventricle (LV) including septum were separated from the right ventricle. The LV was cut into half evenly transversely along the biggest circumstance of the infarcted area. One half was fixed in 2% paraformaldehyde and stained for ß-galactosidase activity as described earlier. After X-gal staining, the hearts were cut longitudinally and embedded in paraffin for H&E staining and histopathological evaluation on whole slice (2-3mm) with infarcted, peri-infarcted and normal tissue of the LV were cut from the other half by visual inspections, fine-minced with scalpel quickly, mixed well, then half

of the tissue mixture was put into a tube with the RNAlater solution (Qiagen), stored at 4°C, the left half of tissue mixture was snap frozen with liquid nitrogen, then stored at -80°C for future use.

10. Total RNA Preparation and First-Strand cDNA Synthesis

Total RNA was isolated with an RNeasy kit (Qiagen, Valencia, CA), including a DNase-digestion step to exclude contaminating DNA following the manufacturer's instructions. Total RNA (10 mg) was subjected to first-strand cDNA synthesis in a 40-mL reaction mixture containing 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl2,1 mmol/L dNTP (Perkin-Elmer Cetus), 0.825-optical density random hexamers (Pharmacia LKB Biotechnology Inc), 40 U of RNAasin (Promega Corp), and 200 U of murine leukemia virus reverse transcriptase (Gibco BRL). The reaction mixture was incubated at 37°C for 60 minutes, heated to 70°C for 5 minutes to denature the reverse transcriptase, and then cooled on ice for 3 minutes. Water (60 mL) was added to each sample, and the samples were stored at -20°C.

11. Quantitative Real-time PCR

Quantitative Real-time PCR was performed to compare the different inflammatory cytokines expression changed. Real-time PCR with relative quantification of target gene copy numbers in relation to ß-actin transcripts was carried out using the following primers:

ß-Actin	F-5' CACGCCATCCTGCGTCTGGA3'
	R-5' AGCACCGTGTTGGCGTAGAG3'
IL-1ß	F-5' TCAGGCAGATGGTGTCTGTC 3'
	R-5' GGTCTATATCCTCCAGCTGC3'
IL-6	F-5' AACGCCTGGAAGAAGATGCC 3'
	R-5' CTCAGGCTGAACTGCAGGAA3'
IL-8	F-5' TTTCTGCAGCTCTCTGTGAGG 3'
	R-5' CTGCTGTTGTTGTTGCTTCTC3'
IL-10	F-5' GCGACTTGTTGCTGACCGG3'
	R-5' GAACCTTGGAGCAGATTTTG3'

The My-iQ system (Bio-Rad) was used to monitor real-time PCR amplification using SYBR Green I (Molecular Probes), a nonspecific, double-strand DNA intercalating fluorescent dye. All reactions were carried out in a total volume of 25 uL with the Takara Ex *Taq* R-PCR Version (Takara Bio, Shiga, Japan). Reaction conditions were the following: hot start for 120 s at 95°C, melting at 95°C for 15 s, annealing at 56°C for 12 s, and amplification at 72°C for 15 s. Reading of the fluorescent product was set to be 2°C below the specific melting peak of the product to eliminate reading of nonspecific products and primer dimers. This was carried out at 85°C for 6 s after each cycle for the above genes. Optimal annealing and melting temperatures were determined for the primers before the running of the samples. Melting temperature analysis for the reaction mix revealed a characteristic melting profile, with a single sharp peak at the typical melting temperature for a given product. Specificity of the product was determined by generation of a melting curve, and gels were run to control for the formation of nonspecific bands. Samples were run in duplicate, and the average crossing point (CP) value was used for calculations. The CP, which is the cycle at which the amount of amplified gene of interest reached a threshold above background fluorescence, was determined to quantitate initial starting copy numbers.

The relative quantity of mRNA expression was calculated with the comparative CP method using the following formula.

Ratio=$(E_{target})CP_{target}$(control-sample)/$(E_{ref})CP_{ref}$(control-sample), where E is the real-time PCR efficiency, CP is the crossing point, and Δ is the difference of a sample vs. control.

The relative quantitation value of a target gene, normalized to ß-actin as the internal control gene, is expressed as a number, which indicates the relative expression compared with that gene. To avoid the possibility of amplifying contaminating DNA and nonspecific amplification, the following precautions were taken: *1*) a DNase digestion step was included in the RNA-extraction protocol, *2*) some primers were designed to include an intron sequence inside the cDNA to be amplified, *3*) reactions were performed with appropriate negative controls (template-free controls), *4*) a uniform amplification of the products was rechecked by analyzing the melting curves of the amplified products (dissociation graphs), and *5*) gel electrophoresis was performed to confirm both the

correct size of the amplification products and the absence of nonspecific bands, respectively.

12. RT-PCR and Gel Bands semi-quantification

To the same samples used in Quantitative Real-time PCR assay, the cDNA also used in Regular PCR to compare the MMP2 and TIMP1 gene expression changes. The PCR reaction mixture contained 150nM of each primer and 1uL of cDNA of each sample produced as described above. We mixed the primers of ß-Actin and TIMP1 together in one tube for each running, and MMP2 was done separately with the following thermal protocol:

(1) 50 °C for 30 min;

(2) 95°C for 15 min ;

(3) 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min;

(4) Final extension step at 72 °C for 10 min.

Primers used for amplification were:

MMP2	F-5' AGGACAAGTGGTCCGCGTAAAG 3'
	R-5' CCACTTCCGGTCATCATCGTAGT3'
TIMP1	F-5' GACTAAGATGCTCAAAGGATTCG3'
	R-5' ATCGCTCTGGTAGCCCTTCT3'
ß-Actin	F-5' CACGCCATCCTGCGTCTGGA3'
	R-5' AGCACCGTGTTGGCGTAGAG3'

The PCR products were loaded onto 2% thidium bromide-stainedagarose gels (1 %) in TBE (9). A 1 kbp DNA ladder molecular weight marker (Life Technologies, Rockville, MD) was run on every gel to confirm expected molecular weight of the amplification product. Images of the RT-PCR ethidium bromide-stained agarose gels were acquired with a Cohu High Performance CCD camera (Cohu Inc. San Diego, CA) and quantification of the bands was performed by ImageJ-1.41(NIH, US). Band intensity was expressed as relative absorbance units normalized to ß-Actin. The ratio between the samples was determined and compared. Mean and standard deviation of all experiments performed were calculated after normalization to ß-Actin.

13. Histopathological analysis

A series of sections (at least 2-3slides/rat) from each heart specimen were stained with hematoxylin and eosin according to the following protocol:

Paraffin-embedded tissue was dewaxed, and then taken into water.

Place sections in hematoxylin for 5 minutes.

1. Wash in tap water.
2. 'Blue' sections in tap water.
3. Place sections in 1% acid alcohol for a few seconds.
4. Wash in tap water.
5. Place sections in eosin for 5 minutes.
6. Wash in tap water.
7. Dehydrate, clear.
8. Mount sections in Permount.

The histological sections were examined by an observer blinded with respect to the treatment regimen, for extent of myocardial tissue injury and the intensity of neutrophils infiltration (the mean of the absolute number of neutrophils from six random high-power fields). The following morphological criteria were used to determine the histopathological damage: score 0, no damage; score 1(mild), interstitial edema and focal necrosis; score2 (moderate), diffuse myocardial cell swelling and necrosis; score3 (severe), necrosis with the presence of contraction bands, neutrophil infiltration and capillaries were compressed; and score 4 (highly severe), wide spread necrosis with the presence of contraction bands, neutrophil infiltration, capillaries compressing and hemorrhage.[18]

14. Collagen Content and Area Measurement

Each heart was transversely cut into 5μm slices. Five consecutive micrometer sections were prepared from different samples of the infarcted area. Sections of each sample were stained with collagen-specific dye Sirius red 3BA in saturated picric acid solution (0.1% solution in saturated aqueous picric acid, Sigma) to allow a clear discrimination between cardiomyocytes and collagen matrix, and then quantified with an image analysis system. Under the staining of Sirius red, collagen fibers appear as red structures, and myocytes

and intramyocardial vessels are yellow. Infarct scar area and the total area of LV myocardium were traced manually in the digital images and measured automatically by the computer by the means of ImageJ-1.41 (NIH, US). The slides were scanned with 1000dpi, and then the pictures were RGB split to green color layer for measurement. Infarct size, expressed as a percentage, was calculated by dividing the sum of infarct areas from all sections by the sum of LV areas from all sections (including those without infarct scar) and multiplying by 100.

15. Statistical Analysis

All data are expressed as mean±SEM. Analysis was carried out by using SPSS statistical package (V16.0, Chicago, IL).

Univariate analysis was used to test the differences of all measurements. When a significant P value, <0.05, was shown for certain variables, post hoc test (LSD and Bonferroni correction) was followed. A value of $P<0.05$ was considered statistically significant.

Chapter III

RESULTS

1. MSCs Culture

MSC were generated by standard procedures and grown for at least three passages in culture. Contaminating hematopoietic cells were depleted during passaging, and MSC were morphologically defined by a fibroblast-like, spindle- shaped appearance. (Figure 1, 2).

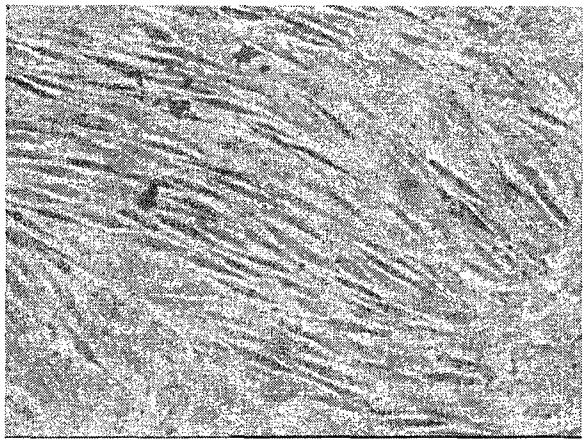


Figure 1

Figure 1 Spindle-shape appearance of MSCs in a culture dish.

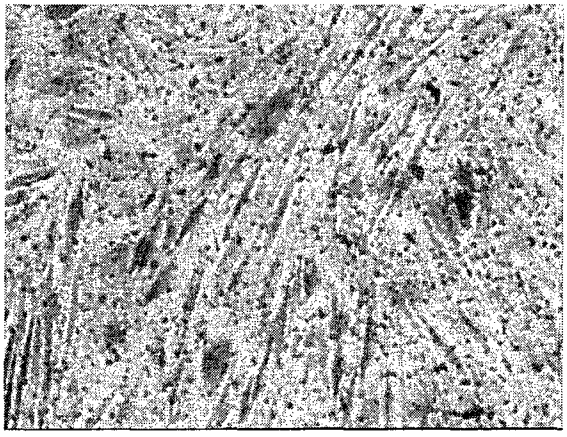


Figure 2

Figure 2 X-gal staining of labeled MSCs (blue color) ready for the transplantation in culture flasks.

2. Mortality and Sample Size

The overall mortality was 15 %(15/103) occurring mainly during the first 48 hours after coronary ligation. No late postopearrative death was observed.

Echocardiographic measurements, histological evaluation and Real-Time PCR were done in all the surviving 88 rats. The number of the rats in each group was distributed as following.

Number of the rats for each group

	12hours	24 hours	1 week	2 weeks
Group L	6	6	6	7
Group M	7	8	8	8
Group C	8	8	8	8

3. Inflammatory Cytokines Gene Expression Changes in Left Ventricle after MSCs-Treatmentt

At a time when significant cardiac protection was clearly established, we sought to further elucidate potential mechanisms through different cytokines secretion. Because MSC are known to have immunomodulatory and paracrine properties, we screened the hearts by quantitative real-time PCR, for changes of pro- (IL-1β, IL-6 and IL-8) and anti-inflammatory (IL-10) cytokines expressions.

The fresh heart tissue slices for the total RNA extraction included infarcted area, peri-infarcted area and normal heart tissue cut from LV by visual inspections, so the gene expression changes stand for the global gene expression changes of the whole LV.

Our pilot experiments have demonstrated that there were no statistical differences between the Group L and Group M, and the focus of our experiment is to explore how the MSCs can improve the cardiac function compared to that of the cell culture medium treated heart. Therefore we showed the data of comparative gene expression in infracted left ventricle treated with MSCs referencing each gene to ß-actin as internal control generated all data.

Compared to the Group M, Group C showed increased gene expressions, including IL-1β, IL-6 and IL-10 from 12 hours to 2 week. For IL-8, it was also up-regulated till to 24 hours, but after that is was down-regulated.

Except for the ratio of IL-1β/IL-10 and IL-6/IL-10 at 1 week, all the other data from pro-/anti inflammatory cytokine gene expression ratios showed significantly inbalance between the pro- and anti-infalmmatory cytokines expression, which meant that the anti-inflammatory effect overwhelmed that of the pro-inflammatory in Group C (Fig 3).

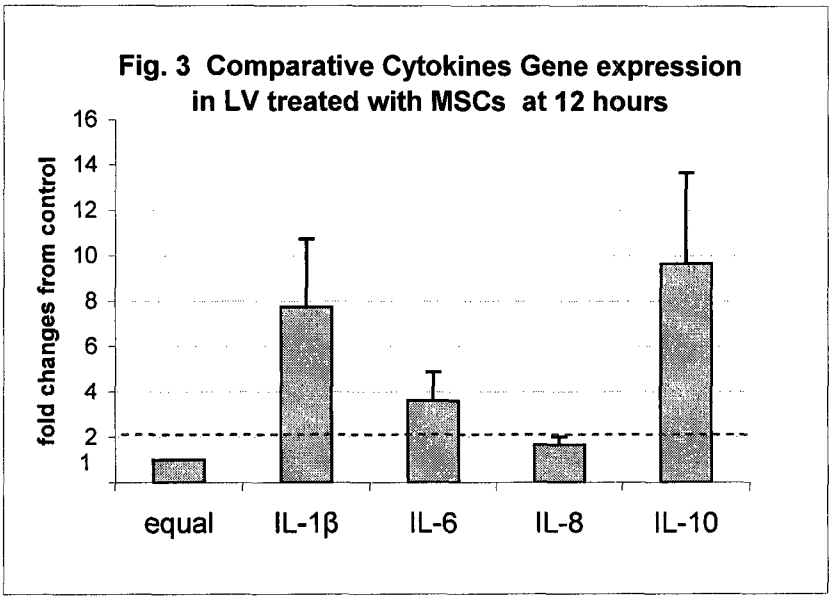


Figure 3 Comparative gene expressions ratios in left ventricule of the hearts treated with MSCs. The data was generated by referencing each gene to ß-actin as internal control.
First bars on all panels depict gene expression ratio of 1, a value obtained when gene expression ratios between MSCs and cell culture medium-treated animals are "equal." Values above or below 1 indicate that gene expression in MSCs-treated animals has increased or decreased compared with cell culture medium-treated animals.
Compared to Group M, Group C caused significant increase (9.6-fold, $P< 0.05$) of anti-inflammatory IL-10 gene expression in the LV. Pro-inflammatory gene expressions of IL-1β (7.7-fold, $P< 0.05$), IL-6 (3.6-fold) and IL-8 (1.6-fold) were also increased in Group C.

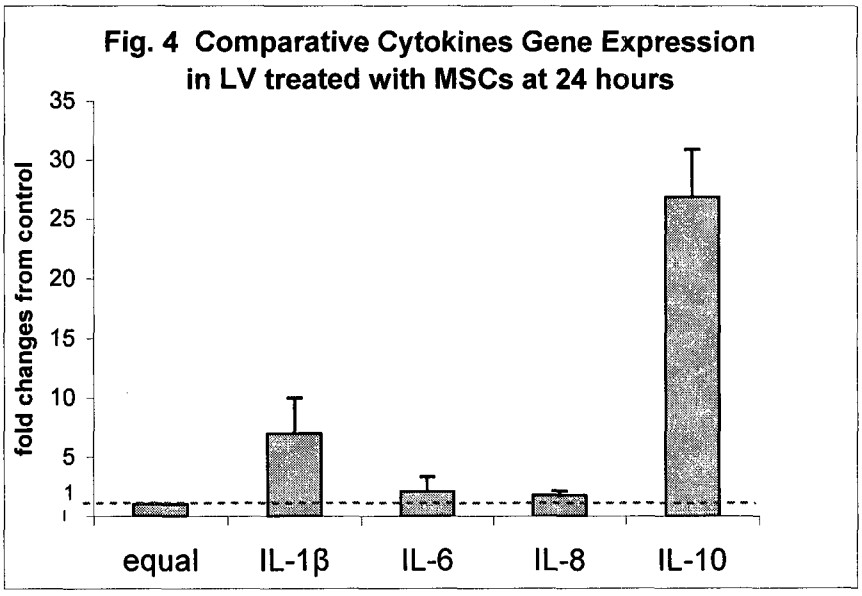


Figure 4 The anti-inflammatory gene expression of IL-10 was robustly expressed in Group C (26.9-fold, $P< 0.05$), and at the same time that of pro-inflammatory ones, IL-1β (6.9-fold, $P< 0.05$), IL-6 (2.0-fold) and IL-8(1.7-fold) also increased in Group C.

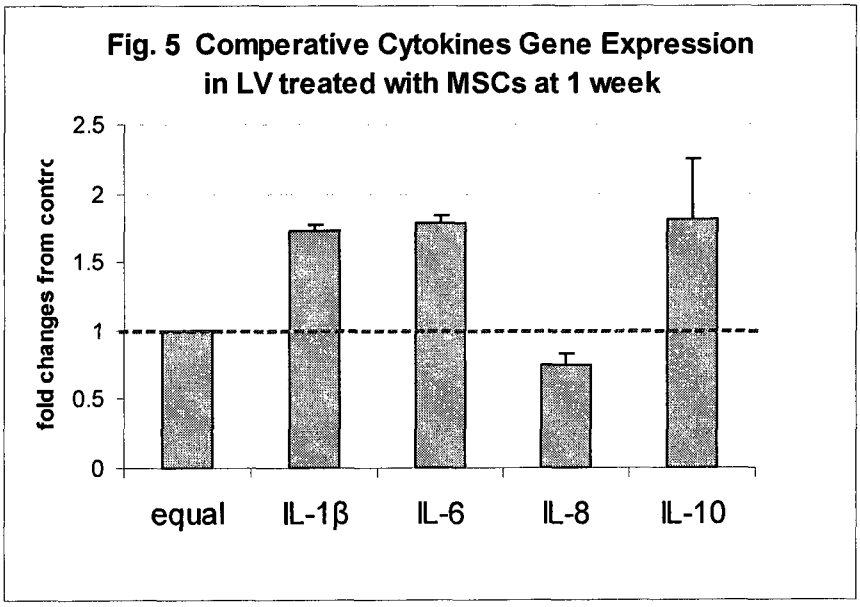


Figure 5 At 1 week after treatment, the comparative gene expressions of IL-10 (1.8-fold), IL-1β (1.7-fold) and IL-6 (1.7-fold) were increased slightly, but did not reach

statistical difference. Pro-inflammatory IL-8 (0.75-fold) was depressed in Group C, however no statistical difference was reached.

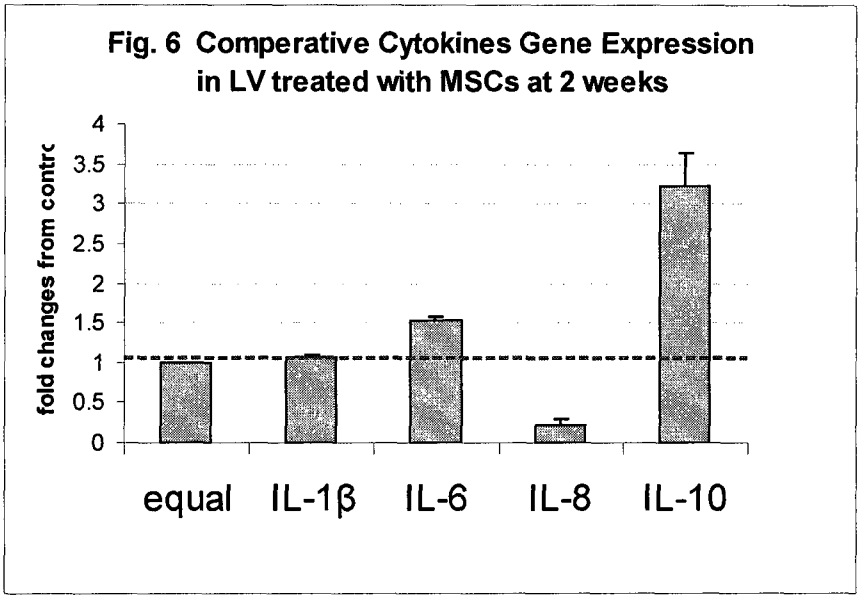


Figure 6 Similar results found at 2 weeks, the comparative gene expressions of IL-10 (3.2-fold), IL-1β (1.1-fold) and IL-6(1.5-fold) were increased slightly in Group C, but did not reach statistical difference. Pro-inflammatory IL-8 (0.2-fold) was depressed more than that at 1 week for Group C.

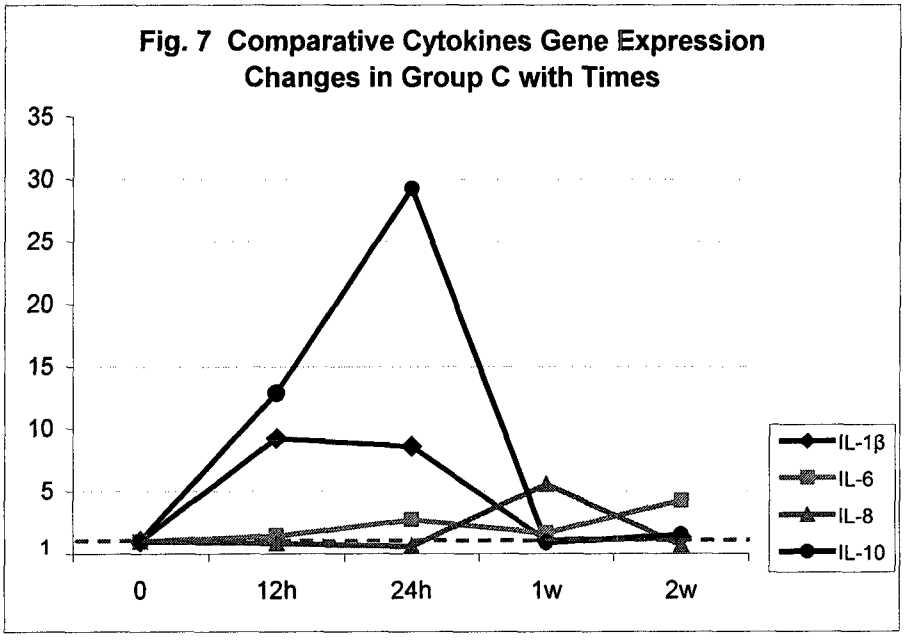


Figure 7 From above, all cytokines experienced most changes around 12-24 hours, while the anti-inflammatory cytokine, IL-10 had an obvious peak around 24 hours.

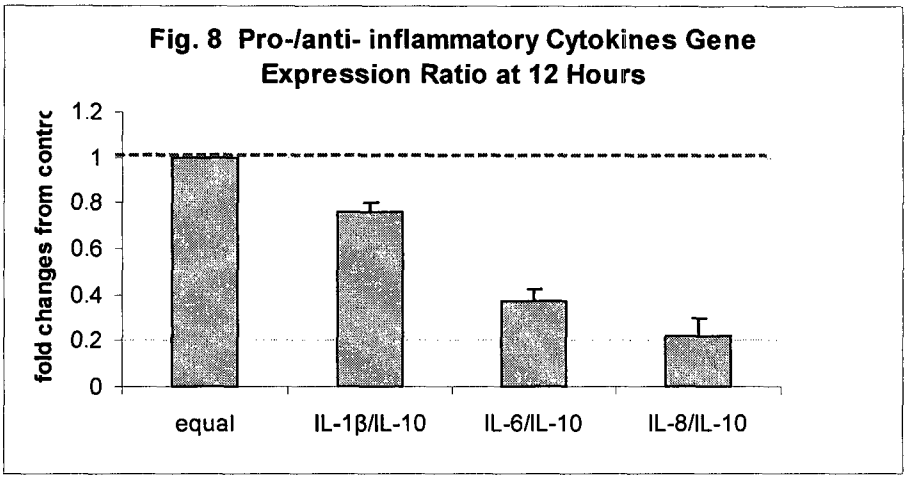


Figure 8 At 12 hours, Group C caused a decrease in the pro-/anti- inflammatory cytokine ratio in the LV, and there were a significant decrease in IL-6/IL-10 (0.37-fold, $P< 0.05$) and IL-8/IL-10 (0.21-fold, $P< 0.05$) rations.

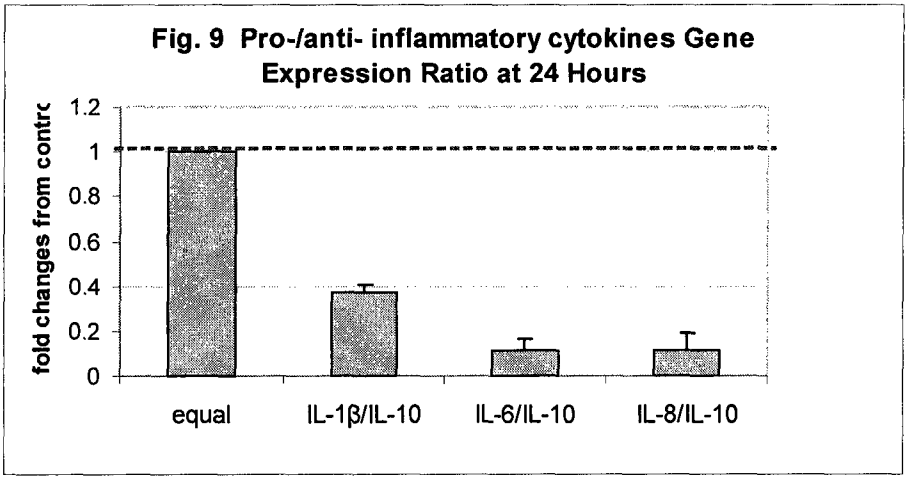


Figure 9 At 24 hours, Group C caused a significant decrease in the pro-/anti-inflammatory cytokine ratio. IL-1β /IL-10 (0.36-fold, $P< 0.05$), IL-6/IL-10 (0.11-fold, $P< 0.05$) and IL-8/IL-10 (0.10-fold, $P< 0.05$).

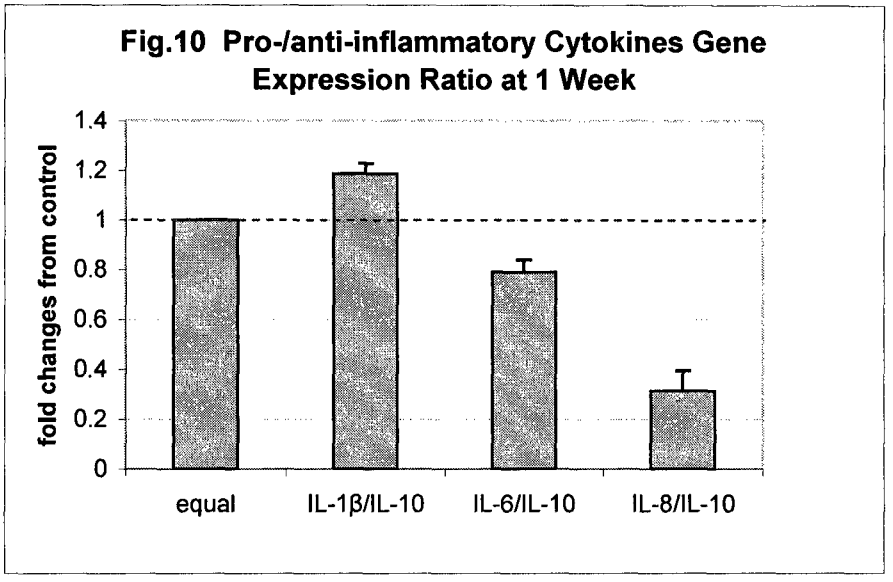


Figure 10 At 1 weeks, MSC treatment caused decrease in the IL-6/IL-10 (0.79-fold) and IL-8/IL-10 (0.32-fold, $P< 0.05$) in the LV. For IL-1β /IL-10, the value of 1.18 means IL-1 β was expressed more than IL-10, but did not reach significant difference.

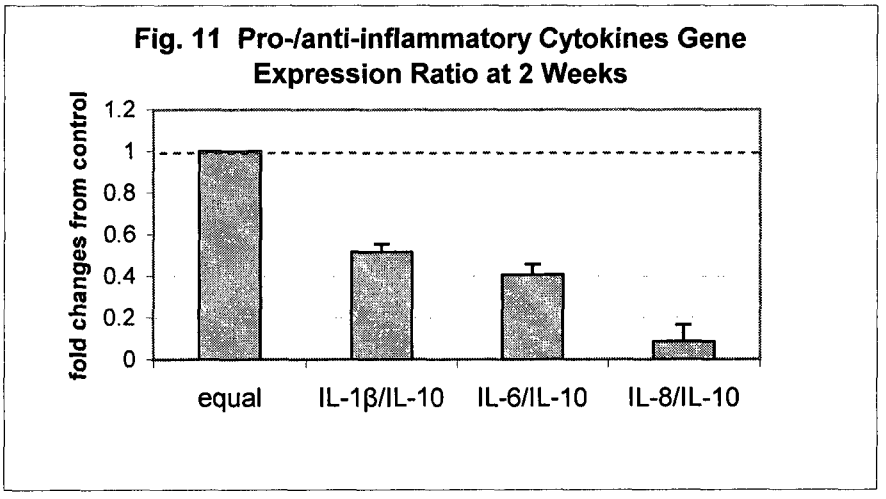


Figure 11 At 2 weeks, MSCs-treatment caused a significant decrease in the IL-1β /IL-10 (0.50-fold, $P< 0.05$), IL-6/IL-10 (0.40-fold, $P< 0.05$) and IL-8/IL-10 (0.08-fold, $P< 0.05$) in the LV.

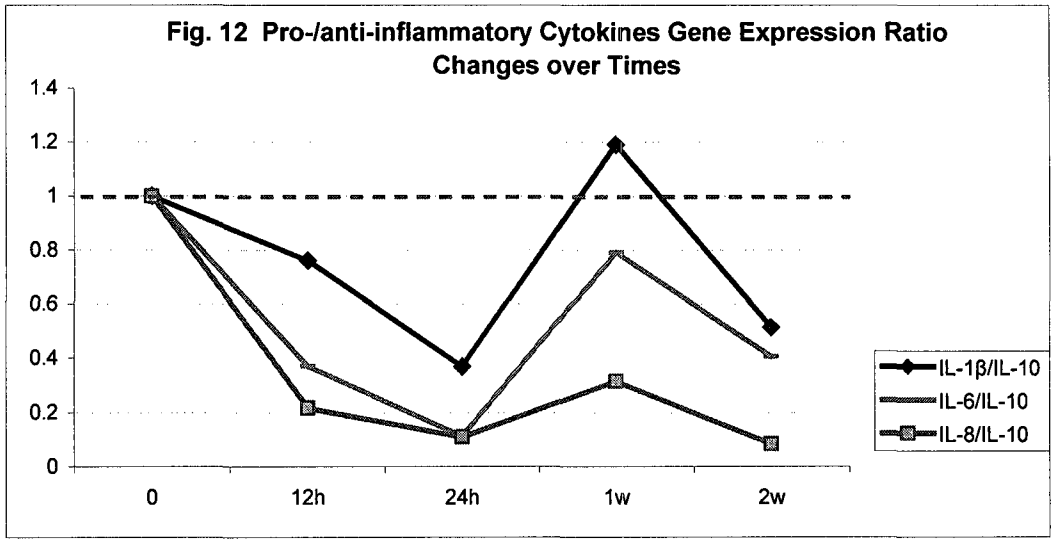


Figure 12 At 1 week after MSC-treatment, all pro-/anti-inflammatory cytokine ratio demonstrated that the anti-inflammatory cytokine, IL-10 was over expressed than that of the pro-inflammatory, except IL-1β /IL-10. This was especially remarked at 24 hours.

4. MMP2 and TIMP1 Gene Expressions in LV

The RT-PCR products of MMP2 and TIMP1 were observed at the expected locations on agarose gels. Intensity analysis indicated that compared to the Group M, Group C caused reduced expression of MMP2 with time (0.50-, 0.57-, 0.95, 0.80-fold), and an increase of TIMP1 at 12 and 24 hours, 1 and 2 weeks (1.24-, 1.09-, 1.02- 2.05-fold) respectively.
The ratio of MMP-2/TIMP1 levels was lower in the Group C at all time frames, reaching statistical significance at 12 and 24 hours.

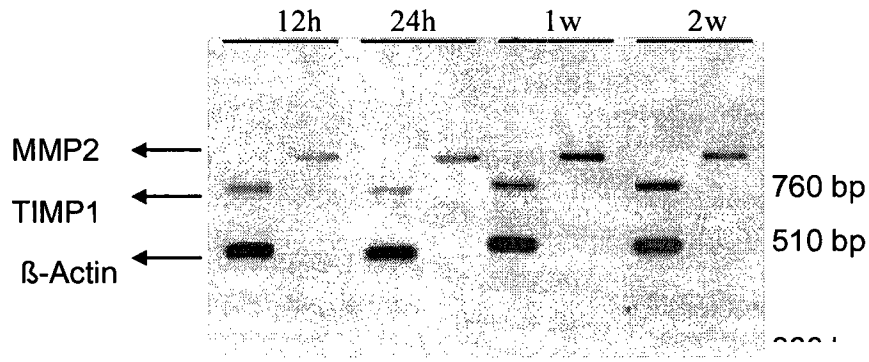


Figure 13 Gene expressions of MMP2 and TIMP1 in LV at various times after MSCs implantation

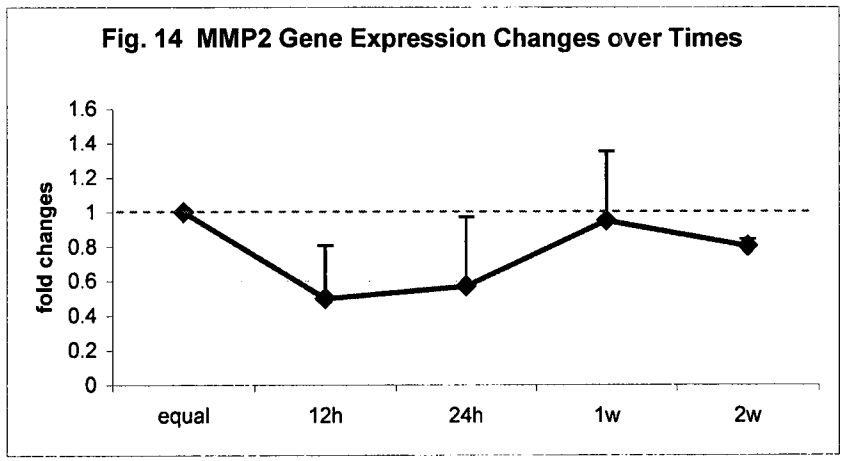


Figure 14 Comparative gene expression ratios in LV of the infracted hearts after MSC- and cell culture medium-treatment. Data generated by referencing each gene to ß-actin as internal control.

Group C caused reduced expression of MMP2 over the time courses (0.50-, 0.57-, 0.95, 0.80-fold).

First points of the graph depict gene expression ratio of 1, a value obtained when gene expression ratios between MSC and cell culture-treated animals are "equal." Values above or below 1 indicate that gene expression in MSC-treated animals has increased or decreased compared with cell culture-treated animals.

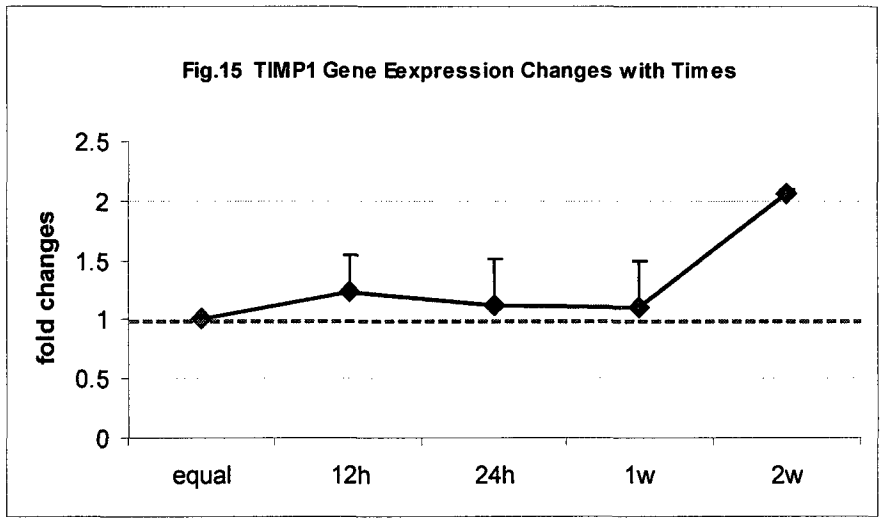


Figure 15 TIMP1 gene expression increased in Group C at 12 and 24 hours, 1 and 2 weeks (1.24-, 1.09-, 1.02- 2.05-fold).

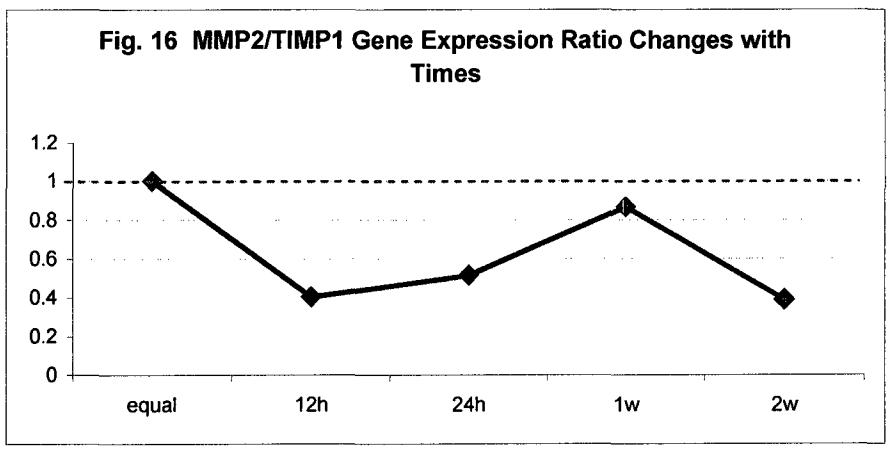


Figure16 MMP-2/TIMP1 levels were lower in the Group C at all time frames, reaching significance at 12 hours (0.40-fold, $P< 0.05$), 24 hours (0.51-fold, $P< 0.05$), and 2 weeks (0.39-fold, $P< 0.05$) respectively.

5. ECM Deposition Measurement

By using Sirius red staining, collagen fibers appear as red structures, and myocytes and intramyocardial vessels are yellow (Figure 17). The computer measured infarct scar area

and the total area of LV myocardium automatically by the means of ImageJ-1.41 (Figure 18).

Histopathological analysis revealed a statistically significant reduction of scar ECM deposition in Group C at 24 hours (C= 0.75 ±0.10% vs M=1.95 ±0.23%, $p<0.01$), 1 week (C=9.36 ±1.24% vs. M=19.30 ±3.63%, $p<0.01$), and at 2 weeks (C= 7.57 ±1.41% vs. M=24.46 ±3.04%, $p<0.01$) (Figure 19).

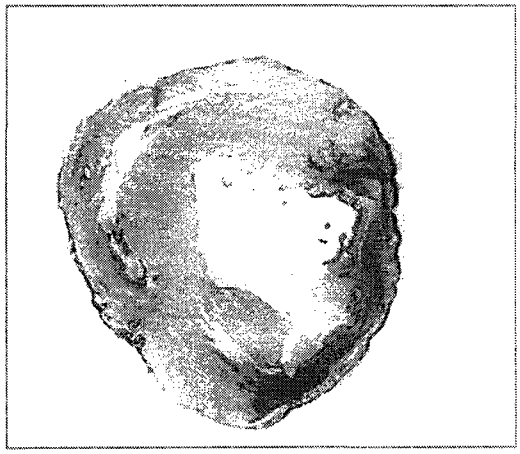


Figure 17 Representative rat heart following left ascending artery ligation at 2 weeks, note that collagen fibers of the scar tissue appeared as red structures, and myocytes and intramyocardial vessels were yellow.

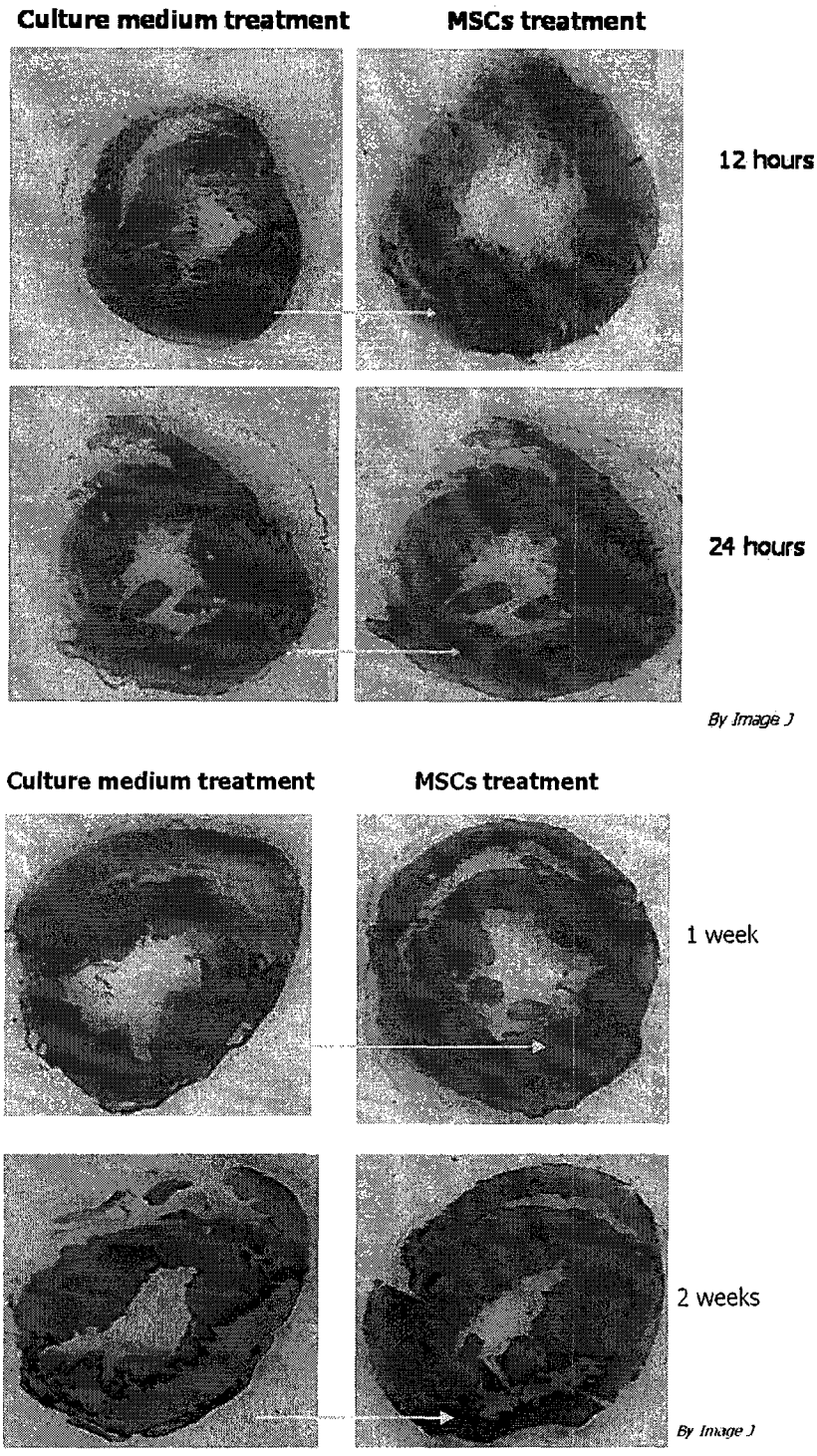


Figure 18 Infarct scar area and the total area of LV myocardium measured automatically by the computer by using ImageJ-1.41 (NIH, US). Note that red area represents ECM

deposition in scar tissue, grey area represents myocardium. Compared to the Group M, Group C had significantly less ECM deposition at each time course.

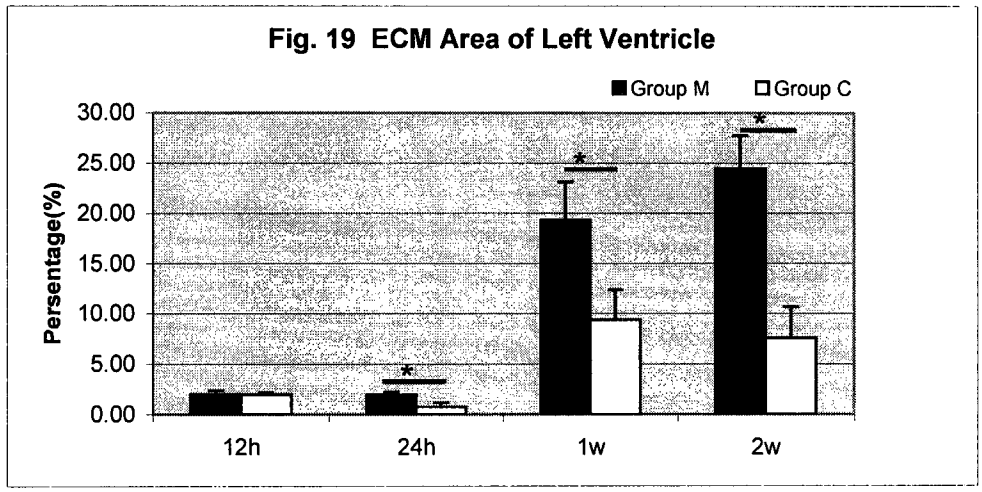


Figure 19 Compared to the Group M, Group C had significantly less ECM deposition at each time course after 24 hours.

6. Histopathological Evaluation and Myocardial Injury score

Histological examination of serial sections of every hearts of Group C confirmed the successful engraftment of MSCs at 12, 24 hours and 1 week after cell implantation, but labeled MSCs were difficult to identify at 2 weeks (Fig. 20). With the H&E staining, the sections were examined by an observer blinded to the treatment regimen. The extent of myocardial tissue injury and the intensity of neutrophils infiltration (the mean of the absolute number of neutrophils from six random high-power fields) were evaluated in a blinded fashion.

Tissue examination of acute myocardial injury and inflammatory cell infiltration at 12 and 24 hours showed significant increase in Group C compared to Groups M ($C=1.73 \pm 0.12$ vs. $M= 21.22 \pm 0.10$ $p<0.05$) and ($C=3.16 \pm 0.51$ vs. $M=1.65 \pm 0.22$, $p<0.05$); Subacute myocardial injury and inflammatory cell infiltration at 1 and 2 weeks showed a significant decrease in Group C compared to Groups M ($C=1.71 \pm 0.10$ vs. $M= 2.15 \pm 0.13$ $p<0.05$) and ($C=0.98 \pm 0.46$ vs. $M=1.70 \pm 0.42$, $p<0.05$)(Figue 21)

Figure 20

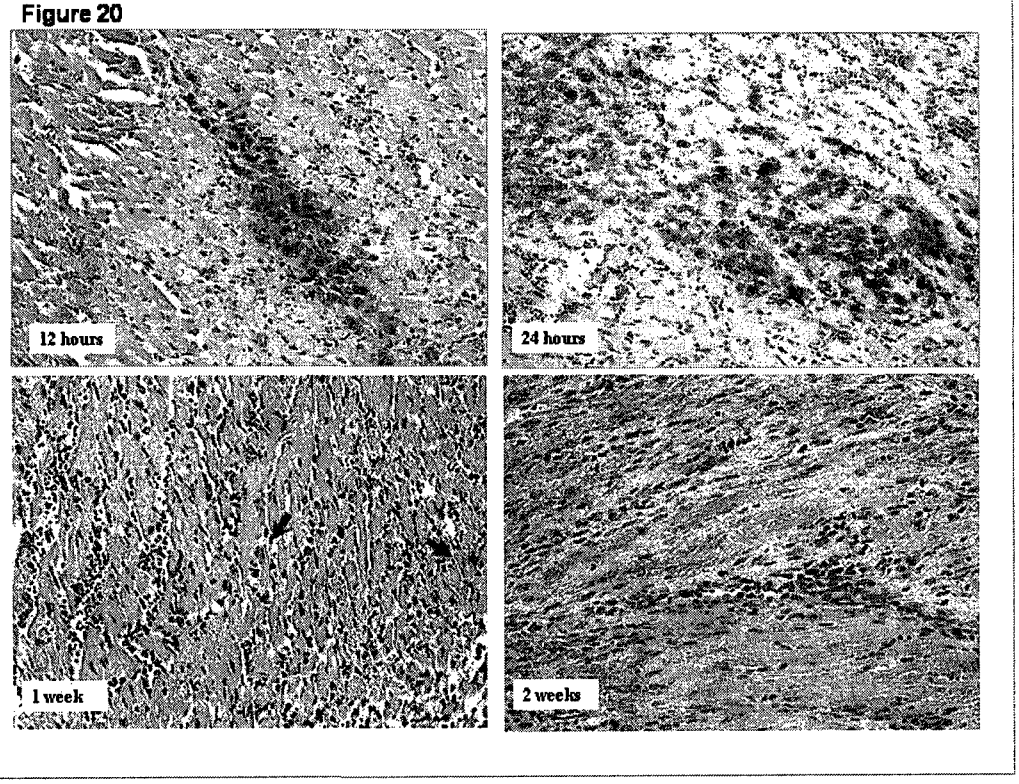
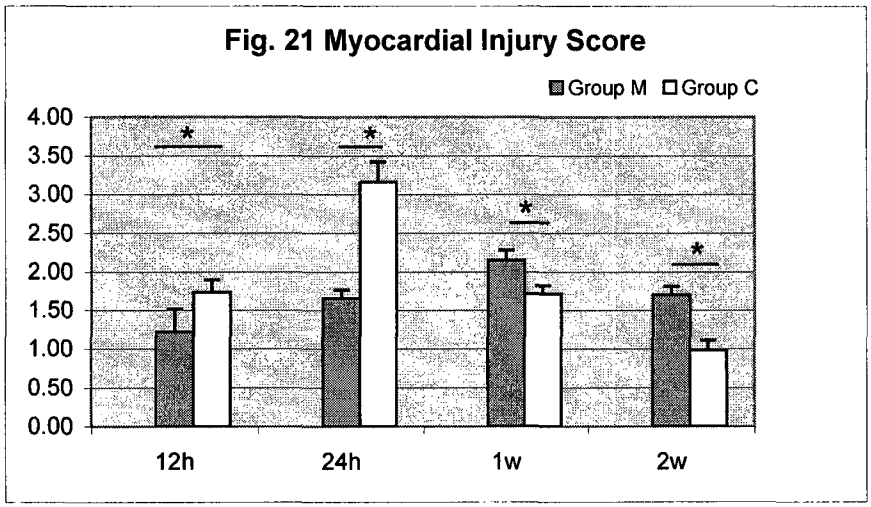


Figure 20 Representative sections(stained with H&E and X-gal) of rat myocardium with evidence of engraftment of allogeneic MSCs harvested at 12 and 24 hours, 1 and 2 weeks after coronary ligation.



Figue 21 Tissue examination for acute myocardial injury and inflammatory cell infiltration at 12 and 24 hours showed significant increase in Group C compared to Group M, however the chronic myocardial injury and inflammatory cell infiltration at 1 and 2 weeks showed a significant decrease in Group C compared to Group M.

7. Cardiac Functional Assessment

Representative echocardiogrphs of normal hearts (A), infracted rat hearts treated with cell culture medium (B) or treated with MSCs (C) were shown; Note the difference in the movement of the anterior wall of LV (Figure 22).

The EF and FS were not significantly different among the groups preoperatively. At 12 hours, 24 hours, 1 week, and 2 weeks, Group C experienced significant improvement in the EF compared with group M (Figure 23), indicating an important beneficial effect of cell therapy. FS was also significantly increased in group C at 24 hours, 1 week, and 2 weeks respectively (Figure 24).

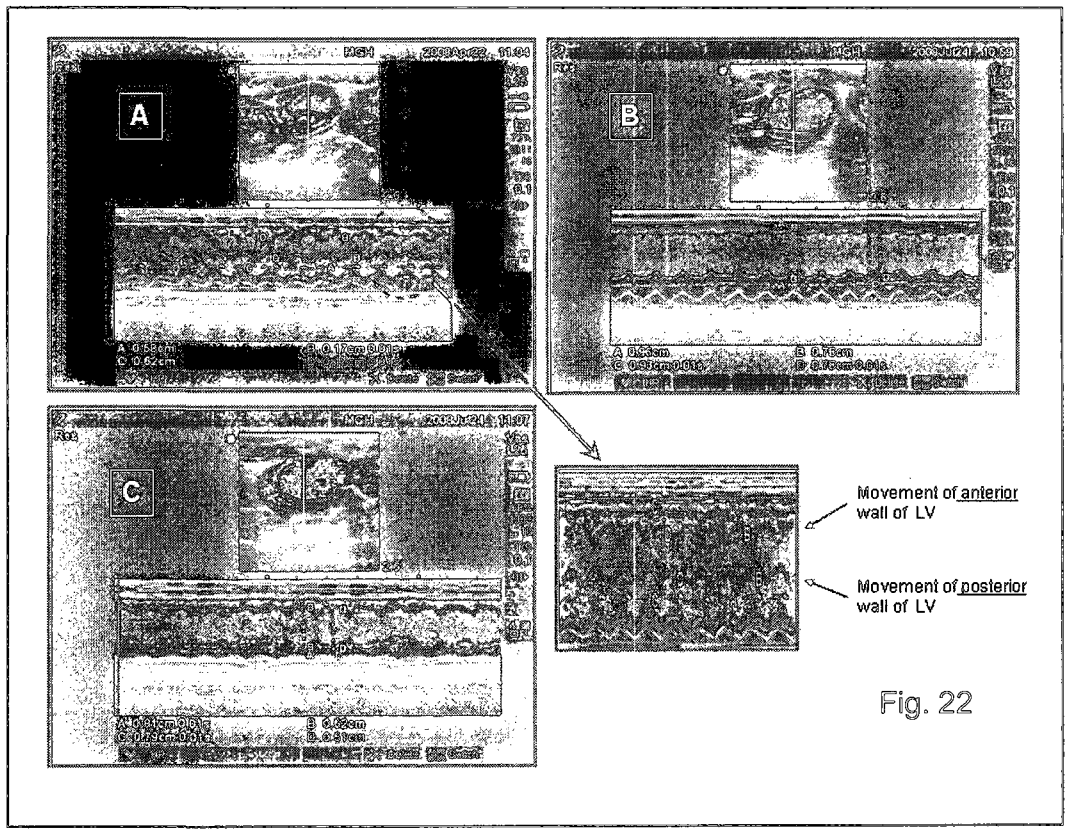


Fig. 22

Figure 22 Representative M-mode echocardiograph performed at 2 weeks after implantation(C). Note the greater movement of the anterior wall of the mesenchymal stem cell (MSC)-injected heart compared with the medium-injected heart (B), and graph A represents normal left ventricular wall movement.

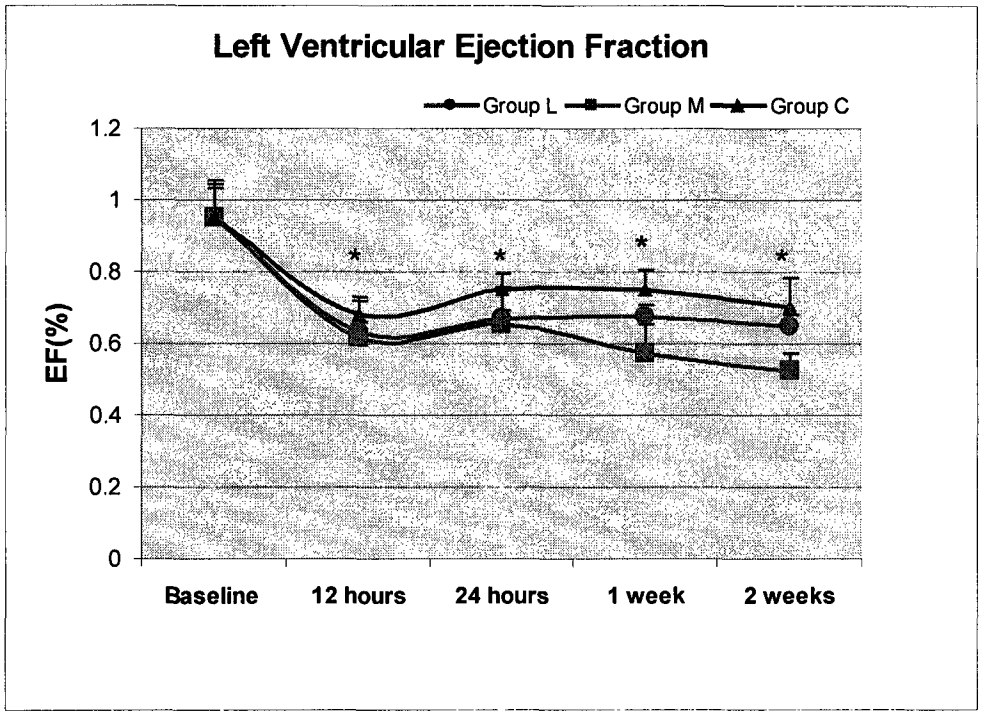


Figure 23 Ejection fractions at various times

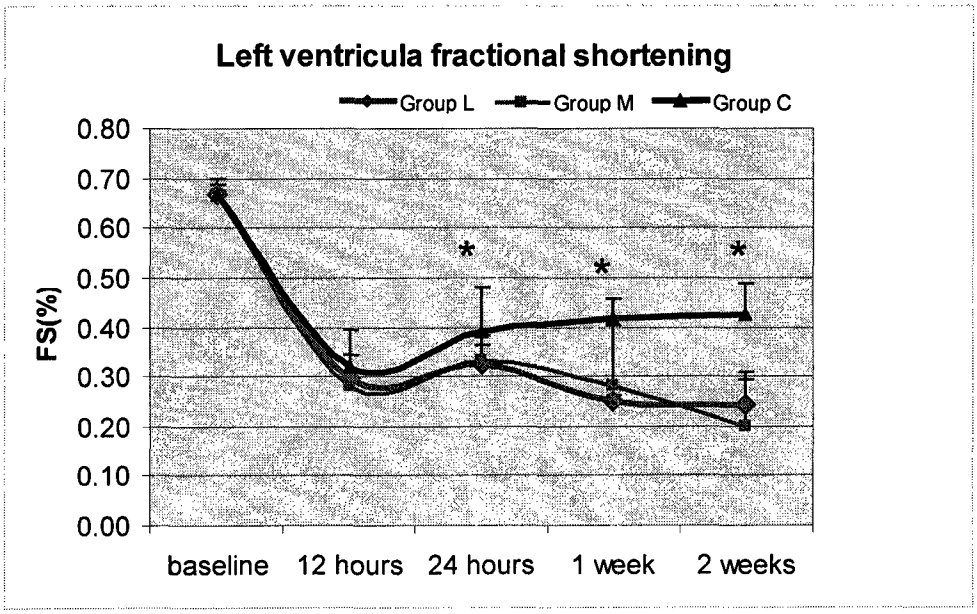


Figure 24 Fractional shortening at various times

Chapter IV

Discussion

Studies from our laboratory and others have already demonstrated the cardiac function improvedby the MSCs transplantation and these MSCs could survive from several days to beyond 6 months [17, 18, and 19]. In this study, we also showed that isogenic MSCs may be an efficient cell source of cell therapy for AMI, as MSC transplantation in Group C showed a significant increase of FS and EF (Fig. 23, 24) as early as 12 hours up to 2 weeks. Although few studies were done to evaluate the cardiac function changes as early as 12 hours after the treatment, Frangogiannis et al found improved cardiac function even from 1 hour after the MSCs transplantation [39].

In our study, the transplanted cells were difficult to be traced from 1 week after the MSCs transplantation (Fig. 20). The low number of donor cells found in the recipient hearts, and the strong therapeutic effect to the infracted left ventricle suggested that the contribution MSCs transplantation might be attributed to other underterminted factors rather than a direct transdifferention of the implanted MSCs into newly formed cardiacmyocyte phenotype. Yukiko [17] also found MSCs disappeared rapidly following implantation. In his first report, they indicated that 90% of transplanted cells were rapidly lost by 15 min after transplantation. Almost all cells were lost in the first 24 hours, and almost complete disappearance had occurred by 28 days post-transplantation. On the other hand, Florian et al [21] observed significant renal function had recovered within 3 days after MSCs therapy, a time period in which none of the administered MSCs had differentiated into kidney tubular or endothelial cell phenotype. Other researchers [22, 23, 24, 25, and 26] have also raised questions over whether stem cells actually differentiate into functioning myocytes and whether the number of engrafted cells could account for the improved function. Tang et al. [27] raised futher doubts over myocardial regeneration leading to functional recovery, because very few engrafted MSCs expressed specific cardiac markers, such as connexin-43 and cardiac troponin I. The small number of engrafted cells suggested that increased myocardial mass as a mechanism for improved cardiac function was less likely [51]. They have attributed the beneficial effects to the secretion of a number of angiogenic factors by the implanted stem cells and the resultant paracrine effects. Other authors have also supported the paracrine theory [28, 29].

Numerous studies have demonstrated activation of cytokine cascades in the infarcted myocardium. Induction and release of the proinflammatory cytokines IL-1β, TNF-α, IL-6,

and IL-8 are consistently found in experimental models and clinical cases of myocardial infarction [30, 31]. Infarct healing is closely intertwined with an inflammatory cascade triggered by hypoxia and cardiomyocyte death [32]. In the past 20 years, a vast body of evidence showed dramatic reduction in infarct size with the use of specific anti-inflammatory strategies [33, 34]. However, attempts to mitigate inflammatory injury in clinical practice have been generally unsuccessful. Emerging evidence suggests that locally delivered MSCs can lead to an improvement in ventricular function, but the cellular and molecular mechanisms involved remain unclear. Unlike other previous studies, our study showed that transplanted MSCs up-regulated both pro- and anti-inflammatory gene expressions, including IL-1β, IL-6 and IL-10 from 12 hours up to 2 weeks; whereas IL-8 was also up-regulated for the first 24 hours (Fig.3-7). This cytokines profile was also contrary to our hypothesis that MSCs might inhibit the pro-inflammatory cytokines expression, while increasing the anti-inflammatory cytokines activity. In a clinical study, Kilic [15] found that the serum concentrations of IL-1β and IL-6 were significantly higher in patients with new coronary events.On the other hand, Florian [21] has shown that at 24 h after injury, the expression of pro-inflammatory cytokines IL-1β, TNF-α and others were significantly reduced and that of anti-inflammatory IL-10 and bFGF, TGF-β, and Bcl-2 were highly up-regulated in treated kidneys.

Even though MSCs have shown to have unique immunomodulatory properties, and can be transplanted as 'universal donor cells' among MHC incompatible individuals, there are still some mild inflammatory reactions at an early stage [40]. Tissue examination of our study also demonstrated that acute myocardial injury and inflammatory neutrophil cells infiltration significantly increased in Group C compared to Groups M at 12 and 24 hours and neutrophil cells infiltration was predominant in 24 hours (Fig 21). These findings suggested us that the transplanted MSCs did not change or inhibit the inflammation reaction histopathologically after acute myocardial. The pro-inflammatory cytokines are mostly produced by the reactive neutrophil cells and damaged endothelial cells of blood vessels, and therefore, it is not expected that MSCs implantation after MI would change this pheonenon. On the other hand, it could reflected the insufficient number of injected MSCs to completely suppress the inflammation, since the majority of the injected cells were lost eaerly after injection, due to mechanical leakage or washout [41].

It has been shown that MSCs could interplay with the cytokine-producing inflammatory cells, and up-regulate the production of anti-inflammatory cytokines, such as IL-10. In our study, IL-10 gene expression has been remarkablely up-regulated and showed evidence of strong cytoprotective effects to cardiac myocytes (Fig 3, 4, 5, 6, and 7). Our results also demonstrated that IL-10 gene expression was associated with the decreased MMP2 and increased TIMP1 gene expression during the repair of diseased heart treated with MSCs (Fig 7, 14 and 15). As MMP-mediated matrix degradation is very crucial in infarct healing and the pathogenesis of left ventricular remodeling, early upregulation of MMP expression might improve the healing myocardial infarcts, and lead to improved LV function [35]. Changes in the cytokine milieu might play a key role in regulating extracellular matrix through inflammatory cells and fibroblasts infiltrating the infarcted myocardium. IL-1ß and TNF-α can increase MMP-2 and MMP-9 activity [36], whereas IL-10 and TGF-a enhance TIMP synthesis, promoting extracellular matrix accumulation, and thus producing a favorable myocardial remodeling for better functional recovery. The analysis of ECM was consistant with reuced scar formation, IL-10 gene expression changes, and the infarcted LV that showed significantly better functional recovery.

Previous studies (37) have demonstrated that early induction of IL-6 was markedly enhanced during the first 6 hours after reperfusion of the previously ischemic myocardium. However, in nonreperfused infarcts, by 24 hours, IL-6 over expression was also observed. Others have suggested that IL-6 mRNA expression was down-regulated after 24 hours of reperfusion in the same ischemic segments in which IL-10 mRNA up-regulation was found. In contrast, animals with permanent coronary occlusion showed that IL-10 mRNA up-regulation and high IL-6 mRNA expression could persist as long as 96 hours [38]. These findings showed the temporal and spatial discrepancies of the cytokines expression during various scenies of myocardial injurys of MI. In our study, both the pro- and anti-inflammatory cytokines gene expression were both up-regulated after MSCs therapy (Fig.7), but the LV functional recovery suggested that the two types of contratory cytokines had imbalanced effects that favor better cardiac functional improvement. After converting the data to ratio of pro-/ anti-inflammatory gene expression, the results showed that the up-regulated anti-inflammatory cytokines gene expression overcame the up-regulated pro-inflammatory cytokines with an overall

upward trend (Figure 12). Therefore the balance between pro-inflammatory and anti-inflammatory cytokines reflected the over-all direction of inflammatory changes after the MSCs treatment. This effect from the MSCs implantation demonstrated that the ratios of pro-/anti-inflammatory cytokines gene expression were in fovor of anti-inflammation and associated with MMP2/TIMP1 ratio, and the size of scar tissue formation that correlated with cardiac functional improvement (Figure 12, 16, 19, 23, and 24). This possible paracrine mechanism of MSC therapy may at least in part explain the early functional improvement after MI following stem cell therapy before transdifferentiation or other mechanisms take place over longer period.

The current study has several limitations. First of all, it is possible that MSCs act through more complex mechanisms through the inflammatory cytokines or other mediators. The currently indentified cytokines may only be the tip of the iceberg. Therefore, further study utilizing knockout animal will be required to clarify the roles of each individual cytokine with respect to the benificial effects of MSCs. Secondly, we did not determin the fate of the transplanted MSCs. We do not know whether the transplanted MSCs furher duplicate or differentiat into functional cells that might replace the damaged cells in the tissue or remained as they were.The mechanism by which MSCs display favorable effect in the infarcted rodent heart model as present in this study could be due to other mechanism such that MSCs facilitated the function of remnant cardiac myocytes cells by restoring the pre-existing stem cell niche in the heart or other undetermined factors. The present study eluded at least part of the mechanism how MSCs improved cardiac functional recovery involving inflammatory cytokined and extracellular matrix.

Chapter V

CONCLUSIONS

In summary, our study showed that isogeneic MSCs injected into peri-infarcted regions of ischemic myocardium after coronory ligation can improve the cardiac function.The ratios of pro-/anti-inflammatory cytokines gene expression favoring anti-inflammation with less scar formation suggested that paracrine mechanism may at least in part explain the early functional improvement. The present findings open up a new and potentially important mode of action for MSCs, which also provides further impetus to study the role of IL-10 as a therapeutic agent.

Reference

1. Merrill CT, Elixhauser A. Hospitalization in the United States, 2002: HCUP Fact Book No. 6. Rockville, MD: Agency for Healthcare Research and Quality; 2005. AHRQ publication 05-0056.
2. Institute of Medicine. Rewarding Provider Performance: Aligning Incentives in Medicare. Washington, DC: National Academy Press; 2007.
3. Centers for Medicare & Medicaid Services Hospital Pay-for-Performance Workgroup. US Dept of Health and Human Services Medicare hospital value-based purchasing plan development: issues paper, 1st public listening session, January17,2007.http://www.cms.hhs.gov AcuteInpatientPPS/Downloads/Hospital_VBP _Plan_Issues_Paper.pdf. Accessed July 17, 2007.
4. Krumholz HM, Parent EM, Tu N, et al. Readmission after hospitalization for congestive heart failure among Medicare beneficiaries. Arch Intern Med. 1997; 157(1):99-104.
5. Gwadry-Sridhar FH, Flintoft V, Lee DS, Lee H, Guyatt GH. A systematic review and meta-analysis of studies comparing readmission rates and mortality rates in patients with heart failure. Arch Intern Med. 2004; 164(21): 2315-2320.
6. Phillips CO, Wright SM, Kern DE, Singa RM, Shepperd S, Rubin HR. Comprehensive discharge planning with postdischarge support for older patients with congestive heart failure: a metaanalysis [published correction appears in JAMA. 2004; 292(9): 1022]. JAMA. 2004; 291(11): 1358-1367.
7. Chiu RCJ, Zibaitis A, Kao RL. Cellular cardiomyoplasty: myocardial regeneration with statellite cell implantation. Ann. Thorac Surg 1995; 60:12-18
8. Friedenstein AJ, Gorskaja U, Kalugina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4: 267-274
9. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation 2001; 104:1046–1052.

10. Besse A, Trimoreau F, Praloran V, Denizot Y. Effect of cytokines and growth factors on the macrophage colony-stimulating factor secretion by human bone marrow stromal cells. Cytokine 2000; 12:522–525.
11. Montfort I and Perez-Tamayo R. The distribution of collagenase in normal rat tissues. J Histochem Cytochem 23: 910–920, 1975.
12. Cleutjens JP, Kandala JC, Guarda E, Guntaka RV, and Weber KT. Regulation of collagen degradation in the rat myocardium after infarction. J Mol Cell Cardiol 27: 1281–1292, 1995.
13. Lindsey ML, Mann DL, Entman ML, and Spinale FG. Extracellular matrix remodeling following myocardial injury. Ann Med 35: 316–326, 2003.
14. Creemers EE, Davis JN, Parkhurst AM, Leenders P, Dowdy KB, Hapke E, Hauet AM, Escobar PG, Cleutjens JP, Smits JF, Daemen MJ, Zile MR, and Spinale FG. Deficiency of tissue inhibitor of matrix metalloproteinase-1 exacerbates LV remodeling following myocardial infarction in mice. 2003 Jan; 284(1):H364-71. Epub 2002 Sep 26.
15. T Kilic, D Ural, E Ural, Z Yumuk, A Agacdiken, T Sahin, G Kahraman, G Kozdag, A Vural, B Komsuoglu. Relation between proinflammatory to anti-inflammatory cytokine ratios and long-term prognosis in patients with non-ST elevation acute coronary syndrome. Heart 2006; 92:1041–1046.
16. Florian To¨gel Zhuma Hu Kathleen Weiss, Jorge Isaac, Claudia Lange, and Christof Westenfelder. Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. Am J Physiol Renal Physiol 289: F31–F42, 2005.
17. Imanishi Y, Saito A, Komoda H, Kitagawa-Sakakida S, Miyagawa S, Kondoh H, Ichikawa H, Sawa Y. Allogenic mesenchymal stem cell transplantation has a therapeutic effect in acute myocardial infarction in rats. J Mol Cell Cardiol. 2008 Apr;44(4):662-71. Epub 2007 Nov 13.
18. Amado LC, Saliaris AP, Schuleri KH, St John M, Xie JS, Cattaneo S, et al. Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells aftermyocardial infarction. Proc Natl Acad SciUSA2005; 102:11474–9.

19. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001; 410: 701–5.
20. Atoui R, Asenjo JF, Duong M, Chen G, Chiu RC, Shum-Tim D. Marrow stromal cells as universal donor cells for myocardial regenerative therapy: their unique immune tolerance. Ann Thorac Surg. 2008 Feb; 85(2):571-9.
21. Florian To¨gel Zhuma Hu Kathleen Weiss, Jorge Isaac, Claudia Lange, and Christof Westenfelder. Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. Am J Physiol Renal Physiol 289: F31–F42, 2005.
22. Dai W, Field LJ, Rubart M, Reuter S, Hale SL, Zweigerdt R, Graichen RE, Kay GL, Jyrala AJ, Colman A, Davidson BP, Pera M, Kloner RA. Survival and maturation of human embryonic stem cell-derived cardiomyocytes in rat hearts. J Mol Cell Cardiol. 2007 Oct; 43(4):504-16. Epub 2007 Jul 14.
23. Suzuki K, Murtuza B, Beauchamp JR, Smolenski RT, Varela-Carver A, Fukushima S, et al. Dynamics and mediators of acute graft attrition after myoblast transplantation to the heart. FASEB J 2004; 18:1153–5.
24. Suzuki K, Murtuza B, Beauchamp JR, Brand NJ, Barton PJ, Varela-Carver A, et al. Role of interleukin-1beta in acute inflammation and graft death after cell transplantation to the heart. Circulation 2004; 110:II219–24.
25. Dow J, Simkhovich BZ, Kedes L, Kloner RA. Washout of transplanted cells from the heart: a potential new hurdle for cell transplantation therapy. Cardiovasc Res 2005;67:301–7.
26. Muller-Ehmsen J, Whittaker P, Kloner RA, Dow JS, Sakoda T, Long TI, et al. Survival and development of neonatal rat cardiomyocytes transplanted into adult myocardium. J Mol Cell Cardiol 2002; 34:107–16.
27. Tang YL, Zhao Q, Zhang YC, Cheng L, Liu M, Shi J, et al. Autologous mesenchymal stem cell transplantation induce VEGF and neovascularization in ischemic myocardium. Regul Pept 2004; 117:3–10.
28. Tang Y, Zhao Q, Qin X, et al. Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. Ann Thorac Surg 2005; 80:229 –37.

29. Besse A, Trimoreau F, Praloran V, Denizot Y. Effect of cytokines and growth factors on the macrophage colony stimulating factor secretion by human bone marrow stromal cells. Cytokine 2000; 12:522–5.
30. Compton SJ, Cairns JA, Holgate ST, and Walls AF. The role of mast cell tryptase in regulating endothelial cell proliferation, cytokine release, and adhesion molecule expression: tryptase induces expression of mRNA for IL-1 beta and IL-8 and stimulates the selective release of IL-8 from human umbilical vein endothelial cells. J Immunol 161: 1939–1946, 1998.
31. Wollert KC and Drexler H. The role of interleukin-6 in the failing heart. Heart Fail Rev 6: 95–103, 2001.
32. Lacraz S, Nicod LP, Chicheportiche R, Welgus HG, and Dayer JM. IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. J Clin Invest 96: 2304–2310, 1995.
33. Koh Ono, Akira Matsumori, Tetsuo Shioi, Yutaka Furukawa and Shigetake Sasayama. Cytokine Gene Expression after Myocardial Infarction in Rat Hearts Possible Implication in Left Ventricular Remodeling. Circulation 1998; 98; 149-156.
34. Paoletti R, Gotto AM Jr, Hajjar DP. Inflammation in atherosclerosis and implications for therapy. Circulation 2004; 109(23 Suppl. 1):III20–III26.
35. Cleutjens JP, Kandala JC, Guarda E, Guntaka RV, and Weber KT. Regulation of collagen degradation in the rat myocardium after infarction. J Mol Cell Cardiol 27: 1281–1292, 1995.
36. Siwik DA, Chang DL, and Colucci WS. Interleukin-1beta and tumor necrosis factor-alpha decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts in vitro. Circ Res 86: 1259–1265, 2000.
37. Alexander Deten, Hans Christian Volz, Wilfried Briest, Heinz-Gerd Zimmer. C ardiac cytokine expression is up-regulated in the acute phase after myocardial infarction: Experimental studies in rats. Cardiovascular Research 55 (2002) 329–340.

38. Frangogiannis NG, Mendoza LH, Lindsey ML, Ballantyne CM, Michael LH, Smith CW, Entman ML. IL-10 is induced in the reperfused myocardium and may modulate the reaction to injury. J Immunol. 2000 Sep 1; 165(5):2798-808.
39. Joseph Francis, Yi Chu, Alan Kim Johnson, Robert M. Weiss, and Robert B. Felder. Acute myocardial infarction induces hypothalamic cytokine synthesis. Am J Physiol Heart Circ Physiol 286: H2264–H2271, 2004;
40. Atoui R, Shum-Tim D, Chiu RC. Myocardial regenerative therapy: immunologic basis for the potential "universal donor cells". Ann Thorac Surg. 2008 Jul; 86(1):327-34. Review
41. Teng CJ, Luo J, Chiu RC, Shum-Tim D. Massive mechanical loss of microspheres with direct intramyocardial injection in the beating heart: implications for cellular cardiomyoplasty. J Thorac Cardiovasc Surg. 2006 Sep;132(3):628-32.