DYNAMIC CHANGES IN HAEMATOPOIOTIC STEM CELLS
AFTER MYOCARDIAL INFARCTION

by

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ABSTRACT

Objective
Increases in the number of CD34+ stem cells and progenitor cells in blood and infarcted areas after acute myocardial infarction (AMI) are a documented phenomenon. However, no study has yet reported on the dynamic changes in specific populations of adult stem cells, such as c-kit + Lin- cells or ckit + Lin - Sca1 + (KLS cells), following AMI. This study investigated the dynamic changes in these cells in multiple systems/organs following MI in mice.

Methods
The C57BL/6J mice received either no surgery (normal control, n=6) or surgical ligation of the left anterior descending coronary artery to create AMI (n=24). On day-1 (n=7), -3 (n=5), -6 (n=6), and -12 (n=6) after AMI, mononuclear cells were isolated from the blood, spleen, and bone marrow, and stained with Lineage-PEcy7, c-kit-PE, and Sca1-APC antibodies. The c-kit + Lin - cell and KLS cell populations in the mononuclear cells were analyzed by FACS flowcytometry.

Results
The pattern of changes in the c-kit + Lin - cells was very similar to that in the KLS cells in the bone marrow, circulating blood, and spleen following AMI. There was a significant increase in these cells on day-3 in the bone marrow (c-kit + Lin- cells: 1.470 ± 0.094% vs control 1.127 ± 0.019 %, and KLS cells: 0.365 ± 0.012 % vs control 0.1848 ± 0.019 %, p<0.05), which then slowly declined from day-6 to -12. In the blood, these cells, particularly the KLS cells, decreased slightly from day-1 to -12. On day-3, -6, and -12 the cells increased continuously and significantly in the spleen, (on day 3, c-kit + Lin-
cells: 0.253 ± 0.0107 % vs control 0.1305 ± 0.014 %; it was 0.3212 ± 0.028 % on day-6). (on day-6 KLS cells: 0.1078 ± 0.076 % vs control 0.0425 ± 0.0064 % while on day 12 it was 0.1174 ± 0.035 % p<0.05).

Conclusion

This study provides for the first time the longest observation of the dynamic changes of specific sub-groups of adult stem cells (c-kit +Lin- cells and KLS cells) in multiple systems following AMI. The study demonstrates that AMI results in significant changes, or mobilization, of these cells in the bone marrow, spleen, and blood. Significant and continuous accumulation of the cells in the spleen occurs following AMI, despite the decreased level of the cells in the blood. The role of the spleen in stem cell mobilization after AMI is unclear and requires further investigation.
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<tr>
<td>AMI</td>
<td>ACUTE MYOCARDIAL INFARCTION</td>
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<tr>
<td>ANOVA</td>
<td>ANALYSIS OF VARIANCE</td>
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<td>ARF</td>
<td>ACUTE RENAL FAILURE</td>
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<td>BM</td>
<td>BONE MARROW</td>
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<td>BMSC</td>
<td>BONE MARROW STEM CELLS</td>
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<td>BrUdr</td>
<td>THYMIDINE ANALOGUE 5-BROMO-2'-DEOXYURIDINE</td>
</tr>
<tr>
<td>CCAC</td>
<td>CANADIAN COUNCIL ON ANIMAL CARE</td>
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<td>CHF</td>
<td>CONGESTIVE HEART FAILURE</td>
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<td>CXCR4</td>
<td>G-PROTEIN-LINKED CHEMOKINE RECEPTOR</td>
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<td>eC-SOD</td>
<td>EXTRACELLULAR SUPEROXIDE DISMUTASE</td>
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<td>EDTA</td>
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<tr>
<td>KLS</td>
<td>C-KIT +,LIN-,SCA-1+</td>
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<tr>
<td>LAD</td>
<td>LEFT ANTERIOR DESCENDING CORONARY ARTERY</td>
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<td>LIN-</td>
<td>LINEAGE NEGATIVE</td>
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<td>MI</td>
<td>MYOCARDIAL INFARCTION</td>
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<tr>
<td>MIP-1A</td>
<td>MACROPHAGE INFLAMMATORY PROTEIN-1 A</td>
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<td>MMPS</td>
<td>MATRIX METALLOPROTEINASES</td>
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<tr>
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<tr>
<td>MNC</td>
<td>MONONUCLEAR CELL</td>
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<tr>
<td>PB</td>
<td>PERIPHERAL BLOOD</td>
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<tr>
<td>PBS</td>
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<td>RBC</td>
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<td>STEM CELL FACTOR</td>
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<tr>
<td>SDF-1α</td>
<td>STROMAL CELL-DERIVED FACTOR-1ALPHA</td>
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<tr>
<td>SEM</td>
<td>STANDARD ERROR OF THE MEAN</td>
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<tr>
<td>SIF</td>
<td>STRESS INTENSITY FACTOR</td>
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<tr>
<td>SM</td>
<td>STAINING MEDIUM</td>
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<tr>
<td>TTC</td>
<td>2,3,5 TRIPHENYL TETRAZOLIUM CHLORIDE</td>
</tr>
<tr>
<td>VEG-F</td>
<td>VASCULAR ENDOTHELIAL GROWTH FACTOR</td>
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DEDICATION

To you, my younger brother, Dr. Al Sadek Elmestiri, who died a year ago, I dedicate this modest effort.

To you, who waited eagerly to congratulate me on my degree.

To you, destined to die from a heart attack as you walked out of your clinic late at night, caring for others as usual, and inattentive to yourself.

To you, who loved me more than yourself, I confess that I love you more than my self and every other being.

To you, who spared no money or effort to support me since I left home.

I swear by God that the tears have not left my eyes as I write these words in your memory.
1 INTRODUCTION

Cardiovascular diseases are the leading cause of morbidity and mortality in the Western world. Myocardial infarction (MI) leads to extensive damage to myocytes and to remodeling of the extra cellular matrix, which, in turn, leads to ventricular dysfunction. Myocardial injury is associated with an intense inflammatory response and healing process, characterized by the release of many growth factors (GF), cytokinase, and chemokinase from damaged tissue, which activate the remodeling process in the heart (Nian, Lee et al. 2004). These growth factors and inflammatory agents also circulate to the bone marrow (BM) to induce dynamic changes in bone marrow stem cells (BMSC) (Kaplan, Psaila et al. 2007).

Recently, a number of studies have addressed the role of adult stem cells, especially hematopoietic stem cells (HSCs), in cardiac tissue repair after acute myocardial infarction (AMI) or other heart diseases. It has been reported that any inflammation, trauma or surgical condition applied to mice, might result in increased levels of stem cells (Fu and Liesveld 2000). Orlic and Kajstura found that HSCs from bone marrow could repair damaged myocytes after AMI when they were transplanted directly into the injured myocardium (Orlic, Kajstura et al. 2001) or mobilized from bone marrow to the heart (Orlic, Kajstura et al. 2001). Adult stem cell mobilization and involvement in microvascular repair has been further demonstrated in a number of experimental and clinical studies (Rajantie, Ilmonen et al. 2004).

For improving myocardial repair and function, adult stem cells from bone marrow can either be directly transplanted into, or mobilized to, the damaged myocardium. However, the major disadvantage of direct transplantation is that the majority of the injected BM stem cells die quickly after local transplantation, although recent studies
have shown that after local injection, preconditioned BMSCs have a better survival rate than non-preconditioned BMSCs. It has been shown recently that mobilization of stem cells from bone marrow to the damaged myocardium can enhance tissue repair following a heart attack. (Tomoda and Aoki 2003). Adult stem cells may improve cardiac function via revascularization, repair of damaged heart cells, or regeneration of myocytes (Canepa, Coviello et al. 2006; Velazquez 2007).

Although there are few studies reporting the changes in stem cells/progenitor cells in a single system following tissue ischemia (Patschan, Krupincza et al. 2006) (Ii, Nishimura et al. 2005), there are no reports on simultaneous dynamic changes in specific sub-groups of adult stem cells in different systems or organs in the same animals following AMI.

The aim of the study was to investigate simultaneous dynamic changes in HSCs in multiple systems/organs in the same animal affected by AMI. The results will lead to a better understanding of stem cell mobilization or activation following AMI and the development of better strategies for cell therapy for myocardial repair following AMI.
2 KNOWLEDGE TO DATE

2.1 Myocardial infarction

2.1.1 Definition

Myocardial infarction is a condition characterized by irreversible necrosis of cardiomyocytes secondary to prolonged ischemia. This usually results from an imbalance of oxygen supply and demand (Gowda, Khan et al. 2003). A prolonged period of myocardial ischemia leads to extensive injury to myocytes and remodeling of the extra-cellular matrix resulting in ventricular dysfunction and heart failure (Sutton and Sharpe 2000).

2.1.2 Epidemiology and prevalence

Acute myocardial infarction and heart failure remain the leading cause of morbidity and mortality worldwide (Thom, Haase et al. 2006). In Canada, for example, congestive heart failure (CHF) affects 400,000 annually resulting in up to 1.38 million hospital days a year. 25 - 40% of these patients will die within one year of diagnosis. In the USA, up to 10 million people have a history of myocardial infarction, angina pectoris or both.

2.1.3 Sequence of changes after myocardial infarction

Following acute myocardial infarction both the infarcted and unaffected myocardial regions undergo progressive changes over the hours, days and weeks following initiation of myocardial Infarction (Rodriguez-Calvo, Tourret et al. 2001). During the first hour heart contractility ceases, followed by the cessation of blood supply to the coronary arteries, subsequently causing necrosis of the sub-endocardium.

In the next 3–6 hours the necrotic region grows outward towards the epicardium, followed by necrosis across the entire ventricular wall (Qin, Liang et al. 2005).
Alterations in the appearance of the infarcted tissue begins about six hours after the onset of cell death. Many abnormalities in cell biochemistry and underlying structures begin occurring within 20 minutes following AMI (Javadov and Karmazyn 2007).

The coagulation necrotic process starts 4-12 hours after the infarction. This is characterized by cell swelling, organelle breakdown and protein de-naturation (Ciulla, Paliotti et al. 2004). Inflammatory cells, including neutrophils and lymphocytes, will enter the infarcted area after 18 hours.

Myocardial infarction triggers all the characteristics of an inflammatory response to initiate the healing process (Vermeiren, Claeys et al. 2000). After 3-4 days, granulation tissue appears at the edges of the infarcted zone; this tissue is responsible for the formation of scar tissue and new capillaries. After 2–3 months, the infarction will have healed, leaving a thin, firm, non-contracting region of the ventricular wall (Burke and Virmani 2007).

2.1.4 Therapeutic strategies

The optimal medical treatments for heart disease, including angiotensin converting enzyme inhibitors, beta-blockers, and aldactone, have shown to decrease symptoms and improve outcomes (Koelling and Eagle 2008). However, clinical trials from 1992 to 2000 have shown that there was no significant decrease in mortality in patients with CHF following these treatments (Lee, Mamdani et al. 2004). Heart transplantation is an effective therapy, but due to the fixed number of donors it is not available to all patients in need (Steinman, Becker et al. 2001). In order to address the increasing incidence of heart failure, stem cell therapy has been proposed as an alternative treatment for heart disease and is currently under extensive investigation (Condorelli and Catalucci 2007).
2.1.5 Rational of developing stem cell therapy

The idea of using stem cell therapy to treat heart disease results from the observation that myocardial infarction leads to structural changes which depress cardiac function (Cleutjens and Creemers 2002). The cardiomyocytes that survive myocardial ischemia primarily respond to cellular hypertrophy rather than proliferation due to the limited mitotic capacity of adult cardiomyocytes (Vandervelde, van Luyn et al. 2005).

Under physiological circumstances this limited mitotic capacity restricts the repair of the ischemic myocardium; ultimately the infarcted tissue will be replaced by fibrotic tissue (Bian, Popovic et al. 2007). The fibrotic tissue can destroy normal contractile function and result in decreased cardiac performance and heart failure. One way researchers are thinking about how to repair the myocardium is to replace lost cardiac tissue with healthy myocardial tissue. A potential source for regeneration of myocardial cells are stem cells.

A therapeutic approach aimed at promoting blood vessel formation (angiogenesis) and the formation of new heart muscle fibers (myocardial regeneration) (Orlic, Kajstura et al. 2001) is a potentially attractive alternative to heart transplantation.

2.2 Stem Cells

2.2.1 Definition and types of stem cells

Stem cells are non-differentiated, immature cells and are also referred to as unspecialized cells. They have the unique ability to divide throughout their lifetime and can evolve into cells with a highly specialized function and take the place of dead or lost cells. This description of stem cells refers to their contribution to the renewal and repair of body tissue (Tuch 2006).
There are two broad categories of stem cell: embryonic stem cells (ESC), which derive from blastocysts, and adult stem cells, which are found in adult tissue. In a developing embryo, stem cells can differentiate into all of the specialized embryonic tissues and in adult organisms they act as a repair system for the body by replenishing specialized cells, but can also maintain the normal turnover of regenerative systems, such as blood and skin (Mimeault, Hauke et al. 2007).

Progenitor stem cells, which are a group of stem cells deriving from primary (immature) stem cells, differ from primary stem cells, which have a reduced differentiation capacity. More importantly, progenitor cells lack the ability for self-renewal, one of the main characteristics of adult stem cells (Morrison, Uchida et al. 1995). Due to ethical issues surrounding the use of embryonic stem cells, the study focuses on adult rather than embryonic stem cells.

2.2.2 Adult stem cells
There are two main groups of adult stem cells: haematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs).

Haematopoietic stem cells have the ability to give rise to all blood cells and are capable of self-renewal (Guo, Lubbert et al. 2003). HSCs may also differentiate to a variety of specialized cells under special conditions by the process of transdifferentiation. (Uher and Vas 2002). Additionally, HSCs are able to mobilize out of bone marrow into circulating blood (Nakano 2003).

Mesenchymal stem cells are multipotent stem cells which can differentiate into a variety of cell types in in-vitro or in-vivo conditions. These cell types include osteoblasts, chondrocytes, myocytes, adipocytes, neuron cells, and pancreatic beta cells (Jiang, Jahagirdar et al. 2002).
2.2.3 Advantages and disadvantages of using adult stem cells

Generally speaking, adult stem cells are limited to differentiating into different cell types of their tissue of origin. However, some evidence suggests that adult stem cell plasticity may still exist (Uher and Vas 2002).

Compared to adult stem cells, large numbers of embryonic stem cells can be relatively easily grown in culture (Wiles and Keller 1991), whereas adult stem cells are usually rare in mature tissues and methods for expanding their numbers in cell culture have not yet been well worked out; this is an important distinction between adult and embryonic stem cells. The difficulty of growing adult stem cells is a disadvantage as large numbers of them are needed for stem cell replacement therapies (Ulloa-Montoya, Verfaillie et al. 2005).

The use of adult stem cells instead of embryonic stem cells avoids some ethical issues. A further advantage in the use of adult stem cells especially in the clinical field is that use of the patient's own adult stem cells ensures that the cells would not be rejected by the immune system. This represents a significant advantage as immune rejection is a difficult problem that can only be resolved with immunosuppressive drugs (Li and Xie 2005).

2.2.4 Haematopoietic stem cells

Haematopoietic stem cells are pluripotent cells that typically reside in the bone marrow and have the capability to differentiate and develop into various blood lineages (Balint, Malesevic et al. 1998). A small population of HSCs remains undifferentiated and self-renews to serve as the source for future blood cell development, as well as being able to transdifferentiate into various tissues. It is estimated that about 1 in 100,000 of whole bone marrow cells are HSCs (Yoshimoto, Chang et al. 2005).
2.2.4.1 Sources of HSCs

A. Bone marrow and peripheral blood

In adults, under steady state conditions, the majority of HSCs reside in the bone marrow. Additionally, HSCs receive their regulatory messages from the microenvironment in the bone marrow. For this reason, the localization of haematopiosis is usually restricted to bone marrow (Heissig, Hattori et al. 2002). However, cytokine and other growth factors (G-F) agents can result in the release of large numbers of HSCs into the blood. The mobilized HSCs in peripheral blood currently replace bone marrow HSCs sourced under clinical conditions because it is easier to harvest peripheral blood (PB) than to harvest bone marrow (Childs, Chernoff et al. 2000).

B. Umbilical cord blood

Since the late 1980s, umbilical cord blood has been recognized as an important clinical source of HSCs. Blood from the placenta and umbilical cord is a rich source of haematopoietic stem cells, cells which are typically discarded after birth (Ebrahim 2002).

2.2.4.2 Identification of HSCs

A. HSC markers

Stem cell markers are given their names based on the molecules that bind to the surface receptors (Civin, Strauss et al. 1984). Previous researchers identified stem cell based on cell size and density (Sutherland, Eaves et al. 1989). However, more recent efforts focus on the absences of cell surface protein markers as defined by monoclonal antibodies (Lineages negative) and presence of other cell surface antigens.
For HSCs in mice, these protein markers include panels of 8 to 14 different monoclonal antibodies that recognize cell surface proteins present on differentiated hematopoietic lineages. Where as the antigen markers are Sca-1 (van de Rijn, Heimfeld et al. 1989), CD27, CD34, CD38, CD43, CD90.1 (Thy-1.1), CD117(c-Kit) (D'Arema, Musto et al. 1998). For example, the cell that has the receptor stem cell antigen -1 on its surface is identified as Sca-1 (van de Rijn, Heimfeld et al. 1989).

Most of the stem cells are usually identified by shorthand, with a combination of marker names reflecting the presence (+) or absence (-) of those markers. None of these markers recognize functional stem cell activity. However, combinations (typically with 3 to 5 different markers) allow for the purification of near-homogenous populations of HSCs. C-kit +, Sca-1+ and lineage negative property, for example, have been identified as the most primitive HSCs in bone marrow ((Chen, Li et al. 2002).

B. C-kit+ and KLS sub-groups

Sca-1 (stem cell antigen-1) is a member of the Ly-6 antigen family expressed on the cell surface of multipotent HSCs. Sca-1 is the most recognized HSC marker in mice with Ly-6 haplotypes (van de Rijn, Heimfeld et al. 1989), whereas c-kit is a membrane-bound tyrosine kinase present on the cell surface of HSCs. The over expression of c-kit has been observed in several stem cells, especially bone marrow HSCs (Dagher, Hiatt et al. 1998). The KLS are those cells that are c-kit positive, Sca-1 positive, and absolutely negative for lineage expression. KLS cells are a population of cells identified as giving rise to life-long myeloid and lymphoid mature cells in mice (Brummendorf, Orlic et al. 2001).

Researchers have found that there are several indications that KLS cells represent a pure population of HSCs. Firstly, over 90% of KLS cells from individual
blast-cell colonies in methyl-cellulose culture were found correlating with very primitive bone marrow activity (Tadokoro, Ema et al. 2007). Secondly, the frequency of KLS cells in the bone marrow (0.01%) is the same as estimates of HSC frequency. Finally, HSC activity has not been found in any population of cells outside the KLS gates (Surdez, Kunz et al. 2005).

For these reasons, KLS cells are considered to be the most common active population of bone marrow HSCs and are invariably mentioned alongside HSCs in this project.

2.2.4.3 Characteristic of HSCs

A. Self-renewal
One essential feature of HSCs is their ability to self-renew, i.e. to make copies with the same or very similar potential (Eaves, Miller et al. 1999). This is an essential property of most stem cells as their presence is essential to the production of the many mature blood cells. However, it is still unclear which key signals allow self-renewal. One link that has been noted is telomerase, the enzyme necessary for maintaining telomeres, which are the DNA regions at the end of chromosomes which protect them from accumulating damage from DNA replication (Stein, Zhu et al. 2004). Expression of telomerase is associated with self-renewal activity; however, the absence of telomerase reduces the self renewing capacity of mice HSCs (Zhu, Zhang et al. 2005).

B. Differentiation
HSCs can differentiate into progenitor and mature adult blood cells. This differentiation, together with the option to self-renew, defines the core function of this type of stem cell
Differentiation is driven and guided by an intricate network of growth factors and cytokines. Differentiation, rather than self-renewal, is the usual outcome for HSCs when stimulated by many factors to which they have been shown to respond (Attema, Paphathanasiou et al. 2007). It appears that once they commit to differentiation, HSCs cannot revert to a self-renewing state. Thus, specific signals, provided by growth factors and cytokines appear to be necessary to maintain HSCs during the organism's lifetime (Veiby, Mikhail et al. 1997).

C. Migration
Migration of HSCs occurs at specific times during development (i.e. during seeding of the fetal liver, spleen and, eventually, bone marrow) and under certain conditions (e.g. cytokine-induced mobilization) later in life (Wright, Wagers et al. 2001). This issue will be discussed in detail in section 2.3.

D. Apoptosis
Apoptosis is a mechanism by which cells can actively self-destruct without causing inflammation. Apoptosis is an essential feature in all multicellular organisms and is a useful mechanism required to regulate HSC metabolism. The effect of apoptosis has been demonstrated in transgenic mouse experiments in which HSC numbers doubled when the apoptotic threshold was increased (Domen 2001).

E. Plasticity
Studies of stem cell transdifferentiation have been highly controversial (Ho and Punzel 2003). In adults, homeostatic cell replacement and tissue regeneration have been
considered to maintain tissue specificity, such as in those tissues and organs that retain stem cell dependence. It is understood that tissue-resident stem cells generate only those mature cell types corresponding to their tissue of origin and do not cross tissue boundaries to generate cell types of different lineages (Bordignon and Roncarolo 2002) but recent experiments have challenged this notion.

The lineage commitment of various adult stem cell populations may, under certain circumstances, transdifferentiate to contribute to a much wider spectrum of differentiated cells than previously expected (Joshi and Enver 2002). Transdifferentiation is the conversion of a cell of one tissue lineage into a cell of an entirely different lineage, followed by a corresponding loss of tissue-specific markers and functions of the original cell type and acquisition of markers and function of the transdifferentiated cell type (Costa and Shaw 2007).

The suggestion that adult stem cells may transdifferentiate has in turn given rise to the concept of stem cell plasticity, which holds that the lineage determination of a differentiating stem cell may not be rigidly defined but is, instead, flexible, thereby allowing these cells to respond to a variety of microenvironmental regenerative signals (Blau, Peterson et al. 1997) (Wagers and Weissman 2004). It has been claimed that haematopoietic bone marrow cells can differentiate not only into blood cells but also into muscle cells (both skeletal myocytes and cardiomyocytes) (Orlic, Hill et al. 2002), brain cells (Bonilla, Alarcon et al. 2002), liver cells (Austin and Lagasse 2003), skin cells (Fathke, Wilson et al. 2004) lung cells (van Haaften and Thebaud 2006) (Kotton, Summer et al. 2003), kidney cells (Lin, Cordes et al. 2003), intestinal cells (Piscaglia, Di Campli et al. 2003) and pancreatic cells (Hess, Li et al. 2003); but until now the mechanism of transdifferentiation has been unclear.
2.2.5 Roles of HSCs in repairing heart and blood vessels

Many studies have shown that HSCs can contribute to myocardial repair (Weissberg and Qasim 2005), but whether HSCs repair myocardium through trans-differentiation, cell fusion, paracrine effect, or other mechanisms is still under question.

Cardiac stem cells, human embryonic stem cells and adult bone marrow stem cells (BMSCs) have been shown to participate in myocardial repair processes and to repopulate the infarcted myocardium (Orlic 2003). Under normal conditions, BMSCs are rarely seen in tissue and organs. However, acute myocardial infarction (AMI) enhances their mobilization into blood circulation and localization in the damaged tissue to repair ischemic myocardium (Wojakowski and Tendera 2005). Moreover, BMSCs can be directly transplanted into the infarcted area by intra-coronary infusion, catheter based intra-myocardial injection, or direct intra-myocardial injection (Leone and Crea 2006). The mobilized or transplanted BMSCs significantly improve left ventricular function, probably due to better regional perfusion and transdifferentiation of stem cells into cardiomyocytes (Rosenstrauch, Poglajen et al. 2005).

2.3 Dynamic changes in haematopoietic stem cells

2.3.1 Dynamic changes of HSCs under physiological conditions

2.3.1.1 Migration of HSCs during early life

It has been confirmed that HSC migration appears to occur at least twice during prenatal development, with both migrations likely to be associated with haematopoietic stem cell expansion (Christensen, Wright et al. 2004).

The first migration involves the colonization of the fetal liver haematopoietic cells (Bonifer, Faust et al. 1998). Prior to their localization in the fetal liver, haematopoietic
stem cells can be isolated from the yolk sac even before completion of the circulatory system (Samokhvalov, Samokhvalova et al. 2007).

The second presumed migration is from the fetal liver to the spleen and bone marrow during embryonic development (Dzierzak, Medvinsky et al. 1998). Haematopoietic stem cells migrate from the fetal liver via blood circulation, enter the spleen and bone marrow (BM), and repopulate this tissue with high levels of immature and maturing cells of all lineages. The mature cells are released into the circulation when needed and at the same time maintain a small pool of undifferentiated (immature) stem cells within the bone marrow during embryonic development. Reportedly, fetal liver HSCs are the precursors of HSCs in adult bone marrow (Kim, He et al. 2006).

Studies have demonstrated that umbilical cord blood cells contain relatively high levels of immature HSC progenitor cells (0.5%), including a minority of more primitive, undifferentiated cells (Lewis 2002). This suggests that high levels of haematopoietic stem cells migrate via blood circulation during the late-stage of embryonic development.

2.3.1.2 Migration of HSCs during adult life

It has been noted that bone marrow serves as the microenvironment that supports adult HSC proliferation and migration. Adult HSCs can migrate spontaneously under special conditions, as in the case of malignant disease, to initiate extramedullary hematopoiesis (Dingli, Mesa et al. 2004). Adult HSCs have also been shown to migrate spontaneously in some genetically altered animals (Wright, Wagers et al. 2001).

Mobilization is a kinetic process by which adult HSCs are made to migrate from the bone marrow into the bloodstream. This process mimics the enhancement of the physiological release of stem cells and progenitors from the bone marrow reservoir in
response to stress signals (Heissig, Hattori et al. 2002). HSCs are probably only occasionally migratory under certain physiological conditions, such as stress and heavy exercise. In adult mammals it is believed that low frequencies of HSCs can be found in the blood due to spontaneous, slow migration of small numbers of HSCs from the bone marrow (Hirayama, Yamaguchi et al. 2003).

As HSCs develop and mature within the bone marrow, blood cells leave the BM microenvironment and egress into the peripheral blood. The majority of undifferentiated HSCs remain in the BM and a small population of the undifferentiated HSCs leave the BM and circulate into the peripheral blood (Pegg 1976); the reason for the release of these HSCs remains unknown. It is possible that the physiological role of these circulating HSCs may be to seed other organs.

2.3.2 Dynamic changes of HSCs under pathophysiological conditions

2.3.2.1 Migration secondary to intrinsic factor “ischemic condition”

Several intrinsic factors play important roles in HSC mobilization from bone marrow niches. Among the important intrinsic factors is the condition of ischemia. It has been reported that after any tissue ischemia there is a release of inflammatory cytokines, growth factors, surface receptors, proteolytic enzymes, and nuclear proteins (Frijns and Kappelle 2002). Different organs such as the heart, brain, kidney, liver and limbs have been shown to recruit stem cells during ischemic conditions.

HSCs have been shown to migrate to areas of injury and subsequently participate in the establishment of the repair process. Physiological triggers alone are often insufficient to mobilize HSCs to induce complete healing in the presence of severe ischemia (Goldstein, Gallagher et al. 2006). During various pathological conditions, particularly ischemia, the trafficking of the HSCs is directed by hypoxic tissue via
hypoxic inducible factor 1-α. (Ramirez-Bergeron and Simon 2001). Ischemia, hypoxia and some cytokines, such as granulocyte macrophage colony stimulating factor (GM-CSF), were known stimuli in the mobilization of HSCs. Multiple mechanisms are involved in the ischemia-induced liberation of HSCs from the bone-marrow pool.

A. Heart ischemia

After a myocardial infarction, the inflammatory response is very active. The degree of intensity of the inflammatory process is important in the healing process (Fuster, Badimon et al. 1992). Usually the infiltrated inflammatory cells secondary to MI are rich sources of cytokines and growth factors that play important roles in the cardiac remodeling process and mobilization of extra stem cells from bone marrow to the site of injury as part of the intrinsic mechanism (de Muinck and Simons 2005). The myocardial growth factors stromal cell-derived factor-1alpha “SDF-1α” expression was seen to significantly increase in the early phase after MI (Wang, Haider et al. 2006). Granulocyte colony stimulating factor (G-CSF) increased by more than 50 times after MI in injured tissues. This factor is known to help mobilize HSCs from bone marrow (Kocher, Schuster et al. 2001). Ischemic cardiac tissue can also secrete vascular endothelial growth factor (VEGF), which also functions as a mobilizer for stem cells from bone marrow (Kalka, Tehrani et al. 2000). These three inflammatory cytokines are not only associated with the regulation of the inflammatory response after myocardial ischemia, but also involved in the induction of mobilized stem cells toward the injured myocardium.

B. Ischemia in other organs

Studies have shown that expression of chemokinase (stroma derived –factors) SDF-1α increases following focal cerebral ischemia. SDF-1α can increase mobilization of BM-
derived cells to damaged areas of the brain, resulting in stimulation of cell repair in the penumbra of the ischemic brain (Shyu, Lin et al. 2007). It is known that endogenous extracellular superoxide dismutase (ecSOD) plays an essential role for post ischemic neovascularization after limb ischemia. In recent studies it has been found that recruitment of inflammatory cells into ischemic tissues and the number of stem cells, such as c-kit$^+$ and CD31$^+$ cells, in both peripheral blood and bone marrow significantly decreased in mice lacking ecSOD$^{-/-}$ after hind limb ischemia (Kim, Lin et al. 2007). In contrast, hind limb ischemia of normal mice stimulates a significant increase in ecSOD activity in ischemic tissues where ecSOD protein is highly expressed at arterioles and in inflammatory cells. This indicates that ecSOD is involved in stem cell mobilization.

Post-ischemic stem cell mobilization was also demonstrated following acute renal ischemia in an animal model. A recent study from Patschan’s group (Patschan, Krupincza et al. 2006) showed that a unilateral renal ischemia in mice for a period of 25 minutes results in transient accumulation of stem cells in the spleen starting within 3-6 hours, but not in peripheral blood during the 7-day follow-up after renal ischemia. This study presents for the first time a chronological analysis of the dynamics of stem cell mobilization in the case of acute renal ischemia.

Other studies have also demonstrated that peripheral blood HSC levels were elevated after extensive liver resection (Dalakas, Newsome et al. 2005) and in patients with alcoholic hepatitis. There is now increasing evidence to suggest that liver injury induces the expression and secretion of signaling mediators, such as SDF-1, IL-8, MMPs, HGF, and SCF, which facilitate the homing and engraftment of HSCs to the liver (Dalakas, Newsome et al. 2005).
2.3.2.2 Migration secondary to extrinsic factors

HSCs or haematopoietic progenitor cells can be mobilized from bone marrow to peripheral blood and other organs in large numbers in response to external stimuli such as the administration of a variety of drugs and cytokines (Velders and Fibbe 2005). There are extensive lists of mobilizing agents that can induce stem cells to migrate from bone marrow to peripheral blood, such as G-CSF (Molineux, Pojda et al. 1990), GM-CSF (Socinski, Cannistra et al. 1988), SIF (Gianni, Siena et al. 1989), IL-1 (102) (Fibbe, Hamilton et al. 1992), IL-3 (Brugger, Bross et al. 1992), IL-7 (Grzegorzewski, Komschlies et al. 1995), IL-11 (Mauch, Lamont et al. 1995), IL-12 (Jackson, Yan et al. 1995), IL-8 (Laterveer, Lindley et al. 1995) and MIP-1a (Lord 1995).

The kinetics of mobilization by these diverse agents varies widely. For example, administration of some agents, such as IL-8 or SIF + G-CSF (Bodine, Seidel et al. 1996), results in rapid (within 15-30 minutes) release of some HSCs into the periphery, whereas for most agents the response takes several days (Morrison, Wright et al. 1997). When the most commonly used mobilizing agent, G-CSF, is given to mice, HSCs expand greatly in the bone marrow prior to mobilization. After the initial expansion phase, HSCs are released abruptly into the blood. Substantially higher numbers of HSCs are released following G-CSF treatment compared to IL-8 treatment (Morrison, Wright et al. 1997).

Chemokines are also important in HSC mobilization (Premack and Schall 1996). For instance, the chemokine stromal cell-derived factor (SDF-I) plays an important role in the mobilization process. Mobilized human CD34 progenitors express reduced levels of the SDF-1 receptor CXCR4, which correlates with improved mobilization, suggesting the involvement of SDF-1/CXCR4 interactions in the mobilization process. Over-
expression of SDF-1 in murine circulation leads to stem cell mobilization (Dar, Kollet et al. 2006).

### 2.3.3 Clinical importance of stem cell mobilization

Mobilization is used as a clinical strategy to increase the source of haematopoietic stem cells for cell transplantation. For example, we can mobilize HSCs from the bone marrow for subsequent collection and transplantation (To, Haylock et al. 1997), as well as for direct seeding of stem cells to injured tissue for repair. Mobilized circulated stem cells are the preferable source of stem and progenitor cells harvested for transplantations to accelerate haematopoietic recovery in patients with acute leukemia because of the higher yield of these mobilized cells, leading to faster engraftment and decreased procedural risks compared with harvested BM cells (Henon, Liang et al. 1992).
3 RATIONALE AND HYPOTHESIS

3.1 Hypothesis

Haematopoietic stem cells either derived from the bone marrow or from other niches participate in tissue repair under certain physiological and pathological conditions (Wagers, Christensen et al. 2002).

It has been suggested that following tissue injury there is a mobilization of stem cells into injured tissue from other parts of the body, such as the bone marrow, and which appears to be triggered by tissue injury. A recent study has shown dynamic changes in stem cell concentration in spleen and peripheral blood circulation following acute renal ischemia, most likely secondary to the mobilization of stem cells from the bone marrow (Patschan, Krupincza et al. 2006). To the best of our knowledge, there have been no publications that report dynamic changes in specific sub-groups of Adult stem cells in bone marrow and peripheral organs following acute myocardial infarction.

We hypothesized that acute myocardial infarction results in significant dynamic changes in some specific sub-groups of Adult stem cells in the bone marrow and other organs. The objective was to investigate the dynamic changes in c-Kit+/Lin- cells and KLS cells in the bone marrow, spleen, and circulating blood following AMI, as well as the relationship of the changes in HSCs between these systems.

3.2 Significance of the study

Congestive heart failure secondary to myocardial ischemia shows an increasing number of cases with no satisfactory treatment. Potential alternative treatments for CHF, such as adult stem cells for cardiac repair and gene therapy, are promising. Adult stem cells
are available in many types of tissue, especially in bone marrow (Ozturk, Guven et al. 2004).

Adult stem cells can be harvested from BM and then injected directly into injured tissue, or they can be induced to enter the peripheral blood by mobilizing factors and subsequently moved into injured tissue. Improvement in heart function has been observed following local injection or mobilization of adult stem cells from BM, however the results are quite controversial (Caplice and Gersh 2003).

The major disadvantage of local injection of BMSCs is that the majority of cells cannot survive in the new environment, and the number of surviving cells is not adequate for tissue repair (van Laake, Hassink et al. 2006).

The purpose of the study was to better understand the dynamic changes in HSCs following acute myocardial infarction, which would provide insight into the potential optimal time and sources for harvesting of mobilizing stem cells after acute myocardial infarction.
4 MATERIALS & METHODS

4.1 Animals
Thirty female mice (C57BL/6J, Jackson Laboratory, Maine, USA) approximately 12 weeks old were used in the experiment and were housed in the animal facility at the University of British Columbia (UBC) under specific pathogen free conditions with free access to foods and acidified drinking water. All animals received humane care in accordance with the guidelines of the Canadian Council of Animal Care (CCAC). The experimental protocol was approved by the Animal Care and Use Committee of UBC.

4.2 Animal modal and surgical procedures

4.2.1 Animal model
Acute myocardial infarction in the mice was created by ligation of the left anterior descending artery (LAD) through a left thoracotomy. The animals were allowed to recover for up to 12 days following the acute myocardial infarction. Tissue samples for stem cell measurements were collected according to the experimental protocol and groups.

4.2.2 Anesthesia and mechanical ventilation
The mouse was weighed and pre-anesthetized with intraperitoneal (IP) administration of a mixture of xylazine/Ketamine in 0.9 % saline. (10 µg/10 mg of body weight) (Figure 1). General anesthesia was induced by inhalation of 3% isoflurane with 30% O₂ for 3-5 minutes in an induction chamber (Figure 2). The hair on the left chest was shaved and ophthalmic ointment was applied to both eyes to protect the cornea during the surgery.

After general anesthesia, the mouse was placed in a supine position on a custom-made device with 45-degree slope (Figure 3). The larynx was exposed by a
curved forceps, and an external light source was placed directly in the animal's neck in order to illuminate the glottis during intubation. A 22-gauge i.v. catheter was carefully inserted into the trachea. After confirming the correct position of the intubation by observing changes in breath patterns by blocking the catheter, the catheter was connected to the ventilator. The animal was ventilated at 120bpm with the tide volume of 200μl. General anesthesia was maintained by inhalation of 1.5~2% isoflurane through a small rodent ventilator (HARVARD Inspira AVS, USA)(Figure 4).

4.2.3 Surgical Procedure
The mouse was fixed in a supine position by taping its legs with the left side elevated (Figure 5). A 1-1.5 cm skin incision was made at the middle-clavicle line of the left chest and parallel to the sternum. After separating the chest muscles, the 3rd and 4th ribs were exposed and cut with extreme caution so as to avoid injury to the internal mammary artery and lung and to allow access to the chest cavity.

The heart was exposed using a small retractor and the pericardium was opened carefully to expose the heart (Figure 6). The left coronary artery and its branches could be identified under a 10X surgical microscope. The left anterior descending artery (LAD) was carefully ligated with a stitch of an 8-0 Nylon suture. A drop of 2% xylocaine was applied to the heart surface to prevent cardiac arrhythmia. Complete occlusion of the LAD was confirmed by the development of an area of pale color with less movement/contraction. After re-expanding the lung, the chest was closed in layers using 6/0 absorbable Vicryl sutures (Figure 7). After spontaneous breath recovered, the mouse was extubated and kept in a recovery cage with a supply of oxygen for about 30 minutes. Analgesia was achieved with subcutaneous injection of buprenorphine (0.025 mg/kg) during the follow-up according to our postoperative care protocol.
4.2.4 Postoperative care

After the surgery, the mice were checked every half hour for the first four hours. Close monitoring was carried out during the early postoperative phase and the health of the animals was assessed daily by monitoring of the surgical scar, body weight, eating, drinking, defecating, urinating, socializing and grooming, according to the monitoring scoring sheet (Figure 8). The mortality rate following AMI in this experiment was around 11% (3 out of 27 mice died or were euthanized before the end of the experiment).

4.3 Animal euthanasia and sample collection

At the scheduled time points according to the protocol and groups, the mice were euthanized for sample collection. Fifteen minutes before euthanization, intraperitoneal heparin (250u/kg, hepalen®, Organon Canada Ltd., Toronto, ON) was given. The mice were deeply anesthetized by intraperitoneal injection of ketamine (100mg/kg) and Xylocain (xylazine 10mg/kg) and inhalation of isoflurane. They were then fixed in a supine position. After opening the abdominal cavity, blood collected slowly from the inferior vena cava and the spleen was then harvested. The femora and tibiae were dissected out and collected in a 60 mm petri dish placed on ice. The heart was harvested and flushed with PBS, followed by staining with 1% 2,3,5-Triphenyltetrazolium chloride (TTC) at room temperature for 20 minutes to identify the infarction. The infarcted area can be easily identified (Figures 9, 10)

4.4 Experimental groups

The animals underwent ligation of the LAD and were sacrificed to collect samples on day-1(n=7), -3(n=5), -6(n=6), -12(n=6) after creating acute myocardial infarction.
Another 6 mice that did not undergo the LAD occlusion procedure were used as normal control.

4.5 Sample preparation for flowcytometry analysis

4.5.1 Blood sample

The heparinized blood sample was placed in a 15 ml conical sterile centrifuge tube and mixed with an equal volume of PBS at room-temperature. The Histopaque 1083 (Sigma) solution was carefully placed in a layer underneath the blood/PBS mixture by placing the tip of the pipet containing the Histopaque at the bottom of the sample tube (3 ml Histopaque per 10 ml blood/PBS mixture). It was then centrifuged for 30 minutes in a GH-3.7 rotor at $440 \times g$ at $18^\circ$ to $20^\circ$C, without a brake. The upper layer containing the plasma and most of the platelets, was removed. The mononuclear cell layer was transferred to another centrifuge tube and the mononuclear cells washed with PBS (the volume was the same as the amount of the mononuclear cell layer) and centrifuged for 5 minutes at $440 \times g$ at $18^\circ$ to $20^\circ$C, repeated three times to remove the platelets as completely as possible.

4.5.2 Spleen

The spleen was placed in a 60 mm petri dish containing 3ml of staining media (SM, PBS with 5% Fetal Bovine Serum and 1mM EDTA) and ground with a pair of glass slides. The cell suspension was filtered through a 70µm cell strainer and the splenocytes were collected in a 15 ml centrifuge tube. Single cell suspension was then obtained.

4.5.3 Bone marrow

Both ends of each bone shaft were cut and the bone marrow was flushed out with 3ml
of PBS using a 5-ml syringe with a 25-G needle. The bone marrow tissue was disaggregated by several passages though the same needle. The supernatant was transferred to a new 15 ml centrifuge tube, which was centrifuged for five minutes at 440 \(\times\) g at 4°C. The supernatant was then discarded.

### 4.5.4 Lysis of red blood cells (RBCs)

The collected cell pellets from the blood, spleen, and bone marrow were centrifuged again. The collected cells were then resuspended in 1ml RBC lysing solution and incubated for 2-4 minutes at room temperature to lyse the RBCs. The collected cell pellets were washed twice by no more than 5 ml of staining media solution, and resuspended with staining media to achieve a final concentration of \(1 \times 10^7\) cells/ml.

### 4.5.5 Immunofluorescence staining

Appropriate controls were prepared to obtain correct results, such as unstained control, isotype antibody stained control, and positive control etc. Briefly, cell suspension in the staining media at a concentration of \(1\times10^7\) cells/ml was achieved after the lysis of RBCs. 100\(\mu\)l of the cell suspension was added to 96-well V-bottom plates, then mixed with 14\(\mu\)l of the pre-diluted labeled antibody and incubated at 4°C in darkness. Lineage-PECy7 (1:400, eBioscience Inc., San Diego, USA), Ckit-PE (1:400, StemCell, Vancouver, Canada), Sca-APC (1:200, eBioscience Inc., San Diego, USA) antibodies were used for the immunofluorescence staining. The cell plates were washed with 2ml staining media and centrifuged at 440 \(\times\) g, 4°C. Finally, the cell pellets were resuspended in 400ul staining media at 4°C for flowcytometry analysis. Data was collected with CELLQuest software on BD FASCAlibus.
4.6 Quantification of hematopoietic stem cells by flowcytometry

A FACS instrument is usually used to sort out the rare stem cells from the millions of other cells. The principle of the methodology has been described previously. Using this technique a suspension of mononuclear cells (MNC), for example, is sent under pressure through a nozzle so narrow that cells can only pass through it one at a time. Upon exiting the nozzle, cells then pass, one by one, through a light source, usually a laser. The system is adjusted so that there is a low probability of more than one cell being present in a droplet.

Just before the stream breaks into droplets, the flow passes through a fluorescence measuring station where the fluorescent character of interest of each cell is measured. An electrical charging ring is placed at the point where the stream breaks into droplets. A charge is placed on the ring-based fluorescence intensity measurement and the opposite charge is trapped on the droplet as it breaks from the stream. The charged droplets then fall through an electrostatic deflection system that diverts the droplets into containers based upon their charge. The fluorescent cells become negatively charged, while non-fluorescent cells become positively charged.

In some systems the charge is applied directly to the stream and the droplet breaking off retains the same charge as the stream. The stream is then returned to neutral after the droplet breaks off. The charge difference allows stem cells to be separated from other cells.

Cells passing through the beam will scatter light which is detected as forward and side scatter (Figure 11). The combination of scattered and fluorescent light is then detected and analyzed. Forward scatter correlates with the cell size, with side scatter
depending on the density of the cells. In this manner, cell populations can often be distinguished based on their difference in size and density.

In this study, quantitative evaluation of c-Kit+/Lin- cells and cKit+/Lineage-/Sca-1+ (KLS) cells was performed using FACS analysis. Representative FACS data are shown in (Figure 11).

4.7 Statistical analyses
Results are expressed as mean ± standard error of the mean (SEM). The means of the groups were compared using one-way repeated measures Analysis of Variance (ANOVA) followed by the Tukey-Kramer post hoc test. Differences were considered significant at $P<0.05$. 
Figure 1. Anesthesia system and induction chamber
Figure 2. Induction of anesthesia in the induction chamber prior to intubation of the mouse.
Figure 3. Setting up for intubation.
Figure 4. Harvard ventilator for mice.
Figure 5. Animal preparation for surgery after intubation.
Figure 6. Exposure of the heart and identification of the LAD anatomy.

Figure 7. Closure of the thoracotomy after ligation of the LAD.
Figure 8. Recovered mouse placed in a new cage.
Figure 9. Sections of the heart with myocardial infarction showing pale color.
Figure 10. Mouse heart showing the infarcted area (pale color) in the left ventricular.
Figure 11. Quantitative evaluation of c-kit+ Lineage-/Sca-1+ (KLS) mononuclear cells by FACS analysis, and representative FACS data obtained from bone marrow, spleen, and blood. Middle panel represents Lineage negative mononuclear cells selected as the lower 5% Lineage staining gated from mononuclear cell region (upper penal). Lower penal represents Lineage negative mononuclear cells with both c-Kit and Sca-1 positive staining that were judged as KLS cells.
5 RESULTS

Dynamic changes in the concentration of c-kit+/Lin- cells and KLS cells in the spleen, bone marrow, and blood were measured by FACS analysis on day-1, -3, -6 and -12 after ligation of the LAD in the mice. Results showed significant changes in these cells in the three systems following AMI.

5.1 Effects of myocardial infarction on c-kit+/Lin- cells

5.1.1 Bone marrow c-kit+/Lin- cells

The percentage of the c-kit+/Lin- cells in the bone marrow significantly increased on day-3 following AMI compared to the baseline level in normal control mice (1.47 ± 0.094% vs 1.127 ± 0.019%, \( P < 0.05 \)). After day-3 following AMI, the percentage of the c-kit+Lin- cells decreased gradually, and returned to the baseline level on day-12 (Figure 12).

![Figure 12. Changes in percentage of c-kit+/Lin- cells in bone marrow at different time points following AMI. * \( p < 0.5 \) vs control (Ctrl).]
5.1.2 Splenic c-kit+/Lin- cells

The percentage of the c-kit+/Lin- cells in the spleen increased gradually following AMI and was significantly higher than the baseline level of normal control mice on day-3, -6, and -12 following AMI. It reached the maximal level (approximately 3 times the baseline level) on day-6 (Figure 13).

![Bar chart showing changes in percentage of c-kit+/Lin- cells in spleen at different time points following MI. * p < 0.5 vs control (Ctrl).]

5.1.3 Blood c-kit+/Lin- cells

There were no significant changes in the percentage of the c-kit+/Lin- cells in the circulating blood at any time point following AMI in ischemic mice relative to the baseline level in the normal control mice. However, there was a trend towards an increase in the percentage of c-kit+Lin- cells only on day-1 following MI (Figure 14).
5.2 Effects of myocardial infarction on KLS cells

5.2.1 Bone marrow KLS cells

The baseline level of KLS cells in bone marrow of the normal control mice was near 0.2% of c-Kit+/Lin- cells. The percentage of KLS cells in the bone marrow slightly decreased on day-1 following AMI, and then significantly increased on day-3 after AMI, reaching more than two times the baseline level of the normal control group (0.3653 ± 0.012 % vs 0.1848 ± 0.019%, P < 0.05). 6 to 12 days following AMI, the percentage of KLS cells slowly decreased, but remained higher than the control group. (Figure 15)
5.2.2 Splenic KLS cells

The baseline level of the percentage of KLS cells in the spleen was five times less than that in the bone marrow in the normal control mice, which was about 0.04% of the c-kit+/Lin- cells. The percentage of KLS cells in the spleen gradually and continuously increased during 12 days following AMI, and was significantly higher on the 6th (0.1078 ± 0.0076 %) and 12th day (0.1174 ± 0.035 %) following AMI than in the normal control mice (0.0425 ± 0.0064 %) (p < 0.05) (Figure 16).
5.2.3 Blood KLS cells

The baseline level of the KLS cells in the blood was similar to that in the spleen in the normal mice. In contrast to the changes in the spleen and bone marrow, the percentage of the KLS cells in the blood decreased gradually during the 12 days following AMI, although there was no statistical significance in the percentage of the KLS cells at any time point compared with the normal control level. On the third day following MI, the percentage of the KLS cells reached the lowest level (Figure 17).
Figure 17. Changes in percentage of KLS cells in blood at different time points following AMI.
6 DISCUSSION AND CONCLUSIONS

A small number of stem/progenitor cells remain in the circulating blood to keep the balance of the stem cell pool in tissue niches that are located in different organs (Fliedner 1998).

The number of stem cells may increase in peripheral blood after so-called pharmacological mobilization (Ratajczak, Majka et al. 2003) (Pituch-Noworolska, Majka et al. 2003) or tissue injury (Ii, Nishimura et al. 2005) (Patschan, Krupincza et al. 2006). Increases in the number of CD34+ stem cells and progenitor cells (EPCs) in the blood and the infarcted area after AMI is a documented phenomenon potentially influencing left ventricular function in the post-infarction setting (Leone, Rutella et al. 2005) and in congestive heart failure (Valgimigli, Rigolin et al. 2004).

However, no study has been reported on the dynamic change in, or mobilization of, more specific sub-groups of HSCs, such as KLS cells, following AMI. The KLS cells are those with c-kit+, Lin-, and Sca-1+. C-kit is a stem cell factor receptor, Sca-1 is a stem cell antigen specifically expressed in various stem cells (only in mice) and Lin is a mixture of antibodies against lineage markers for blood adult cells (mouse: Gr1, Mac-1, B220, CD3 and Ter119; human: CD3, CD4, CD8, CD19, CD33 and Glycophylin A).

Previous studies have confirmed that the presence of the c-kit+, sca-1+ and Lin-markers on a particular stem cell indicates that the cell is BM derived HSCs. Lanza, for example, reported that 70% of BM derived HSCs express the c-kit receptor (Lanza, Moore et al. 2004). Osawa reported that the cells with c-kit+, sca-1+ and Lin- are always purified from murine BM that have long-term multilineage repopulating and contain very primitive HSCs. The data in the study by Osawa et al. clearly indicates that c-kit+, sca-l+ cells are the only cells in the Lin- fraction with HSC activity (Osawa, Nakamura et al. 2004).
Therefore, KLS cells represent a pure population of HSCs, which are found correlating with very primitive bone marrow activity (Surdez, Kunz et al. 2005; Tadokoro, Ema et al. 2007). KLS cells are considered to be the most common active population of bone marrow HSCs. This study attempted to answer the question of whether acute myocardial infarction related stress also triggers dynamic changes or mobilization of KLS cells and c-kit+/Lin- cells from the BM into the peripheral blood and spleen.

This study presents for the first time a chronological observation of the dynamic changes in specific sub-groups of HSCs in the bone marrow, spleen, and circulating blood in the same animal following acute myocardial infarction. Significant dynamic changes in the percentage of c-kit+/Lin- cells and KLS cells in the bone marrow, spleen, and circulating blood during the first 12 days after AMI by ligation of the LAD were clearly demonstrated.

The most interesting findings show that:

1) in the bone marrow these cells slightly decrease in number on the first day after AMI, peak on the third day, and remain elevated between day-6 and -12;
2) there is a continuous and significant increase in the number of these cells in the spleen during the first 12 days following AMI;
3) there is a trend towards a decrease in cell numbers, particularly KLS cells, in the circulating blood during the first 12 days following AMI;
4) the most significant increase in the number of these cells was observed in the spleen; and in BM on the 3rd day following AMI (supplementary Figures 1, 2).

The decrease in HSCs in the bone marrow at the very early stage following AMI is probably due to initial release of existing stem cells from bone marrow to the
circulating blood and organs. After this stage a significant amount of new stem cells are produced in the bone marrow due to a stimulation of some undetermined factors that are most likely activated by AMI or released from injured tissue. This stimulation appears more significant during the first three days following AMI as the HSC level reaches maximal in the bone marrow on day-3 after AMI. The HSC level then decreases gradually and almost returns to the baseline level on day-12 following AMI, which may indicate a gradual decline in the stimulation of stem cell production in bone marrow and/or an increase in the release of stem cells from bone marrow to circulating blood and spleen.

Although the mechanism of stem cell mobilization following AMI has not been completely confirmed, it has been reported that ischemia induces the liberation of stem cells from the bone marrow pool by multiple mechanisms; many mobilizing factors/cytokines, such as G-CSF, SDF-1, CSF, or VEGF, are involved in the stem cell mobilization and homing.

Leone (Leone, Rutella et al. 2006) has demonstrated that the spontaneous mobilization of CD34+ cells into the peripheral blood of patients with AMI is significantly correlated to endogenous G-CSF. G-CSF is a well-known potent mobilizer of CD34+ cells into peripheral blood and is currently widely used for transplantation of haematopoietic progenitor cells, instead of the whole bone marrow as in current clinical practice.

More attention has been devoted recently to the role of the chemokine stromal cell-derived factor-1 alpha (SDF-1α) and its receptor CXCR4 in the trafficking and homing of human stem/progenitor cells. Interestingly, SDF-1 alpha is transiently up-regulated at the mRNA level early after myocardial infarction and might contribute to
intralesional stem/progenitor cells homing (Askari, Unzek et al. 2003). Cumulative evidence has suggested the pivotal role of the interplay between the systemic mobilizing effect of G-CSF and the local contribution to engraftment of SDF1-alpha.

It was shown in this study that the percentage of HSC cells in the bone marrow reaches a maximal level around day-3 following AMI, which is consistent with the findings of Wright (Wright 1985; Wright, Cheshier et al. 2001), who found that the uptake of BrdUr by BM usually occurs 2-3 days following the administration of Growth Factors (G-F). HSCs incorporate BrdUr into its DNA, which reaches the maximum level three days after administration of G-F. Observations made in this study also agree with Barcew's finding that the percentage of HSCs in BM peaks on day-2, but the absolute cell number reaches its peak on day-3 following activation by G-F (Barcew, Kacinska et al. 2004).

The decreased level of HSCs in the BM on day-6 following AMI is probably due to a significantly increased release of stem cells from the bone marrow to circulating blood and other organs. Levesque has observed that the stem cell release from the bone marrow is probably activated by increased numbers of enzymes called serine proteases (Levesque, Liu et al. 2004).

Serine proteases, including enzymes such as casthepsin and elastin, are released upon the activation of neutrophili in the BM after stimulation of G-F and other inflammatory agents. The enzymes have been noted to reach the maximum level of concentration around day-5 or -6. They disrupt the cell surface interaction between stem cells and the microenvironment of the BM, which allows the stem cells to be released to their respective niches and transmigrate across the endothelium layer into the peripheral blood and other organs (Levesque, Hendy et al. 2003).
The decreased HSC level in bone marrow on day-6 corresponds to the significant increase in HSCs in the spleen observed from day-6 to day-12 following AMI, which further supports the probability of significantly increased release of stem cells from BM on day-5 to -6 following AMI. Although the subsequent decrease in HSC level in the BM from its peak level on day-6 is most likely attributed to the mobilization from BM to the circulating blood and other organs, it could also be due to the subsequent development of these stem cells and early progenitors to mature blood cell lineages to help in the recovery of the whole population of bone marrow cells (Nienaber, Petzsch et al. 2006).

Another interesting finding of this study is that the percentage of HSCs (KLS and c-kit+/Lin- cells) fails to increase in circulating blood from day-1 to day-12 following AMI, but also shows a trend towards decrease during the first 12 days. In contrast to the change in the blood, the percentage of these cells in the spleen increases continuously and significantly during the first 12 days, particularly after day-3, following AMI. These findings indicate that the majority of mobilized HSCs in the circulating blood from the bone marrow are either actively or passively removed by the spleen and/or possibly migrate into the injured tissue for tissue repair.

It is unclear whether the spleen plays a role simply as a potential transient reservoir of mobilized HSCs or as an important organ actively participating in stem cell mobilization, trafficking, and activation. The accumulation of the mobilized stem cells in the spleen may play an important role in the late phase of stem cell migration into the injured tissue for the process of continuous tissue repair. Early tissue-injury-induced mobilization of stem cells from the bone marrow decreases after the acute phase of MI.
The phenomenon of a lack of the increase in HSCs in circulating blood following AMI is consistent with findings of Patschan on EPCs following renal ischemia (Patschan, Krupincza et al. 2006). They found that a 25-minute period of unilateral renal ischemia resulted in no increase in the number of peripheral circulating EPCs at 10 minutes, 3, 6, 24 hours, and 7 days following renal ischemia.

The lack of increase in HSCs or EPCs in circulating blood may be due to the existence of a dynamic equilibrium between circulating and tissue-residing HSCs or EPCs characterized by a slow and continuous cell migration from the bone marrow or other niches via peripheral blood to the spleen and later on to sites of ischemic tissue (Levesque, Hendy et al. 2003).

Patschan demonstrated also a significant transient accumulation of EPCs in the spleen occurs only between 3–6 hours following renal ischemia, which is different from this study’s findings, which showed that the significant accumulation of HSCs in the spleen continues after 3 days following AMI. This difference in findings could be due to different groups of cells studied and the ischemic model of different organs. Alternatively, in the study an early peak of accumulation of HSCs in the spleen during the first day following AMI could have been missed as the cells were not measured during the first day.

Hematopoietic stem cell mobilization (migration) is a commonly used practice in the clinical setting to increase the number of HSCs in the peripheral blood for subsequent collection and transplantation (Mayhall, Paffett-Lugassy et al. 2004). HSCs have in particular been extensively used in the process of repair or regeneration of damaged tissue in the heart, kidney, brain and skin—mostly through transplantation of cells harvested from the bone marrow. However, it has been found that the results of
direct transplantation of bone marrow stem cells are not satisfactory because the numbers of harvested cells are inadequate (van Laake, Hassink et al. 2006), or the majority of transplanted stem cells die soon after transplantation. This study provides the dynamics of HSCs following AMI in the circulating blood, spleen, and bone marrow, which may help in developing better therapeutic strategies that augments the naturally occurring repair process following AMI through mobilization of stem cells, and in identifying better sources of HSCs and/or a better time for harvesting and transplantation.

In conclusion, our study provides for the first time the longest observation of the dynamics of specific sub-groups of adult stem cells (c-Kit+/Lin- cells and KLS cells) in multiple systems following AMI. It was demonstrated that AMI results in significant changes in, or mobilization of, these cells in the bone marrow, spleen, and blood. Significant and continuous accumulation of the cells in the spleen occurs following AMI, despite the decreased level of the cells in the blood. The role of spleen in stem cell mobilization after AMI is unclear and requires further investigation.
REFERENCES


Ratajczak, M. Z., M. Majka, et al. (2003). "Expression of functional CXCR4 by muscle satellite cells and secretion of SDF-1 by muscle-derived fibroblasts is associated with the


APPENDIX

Supplementary Figure 1 KLS cells in three systems

Supplementary Figure 2 C-kit+ Lin- cells in three systems
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ANIMAL CARE CERTIFICATE

Application Number: A07-0116

Investigator or Course Director: Jian Ye

Department: Surgery

Animals:

- Mice wild-type C57 Bl6 96
- Mice GFP 180

Start Date: May 1, 2007

Approval Date: May 24, 2007

Funding Sources:

Funding Agency: Canadian Institutes of Health Research (CIHR)

Funding Title: Near-Infrared, thermal and magnetic resonance imaging in diagnostics and treatment of cardiac ischemia and infarction

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.