A COMPARISON OF HAEMATOPOIETIC PROGENITOR CELLS ENUMERATION USING SYSMEX HAEMATOLOGY ANALYZER AND STANDARD FLOW CYTOMETRY.

BY

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6. LIST OF ABBREVIATIONS

ABSCT   Autologous blood stem cell transplant

ABMT   Autologous bone marrow transplant

ACAS   Adaptive Cluster Analysis System

AUC   Area under the curve

BSC   Blood stem cells

CBC   complete blood count

cDNA   cytoplasmic Deoxyribonucleic acid

CD   Cluster of Differentiation

CI   Confidence interval

CFU-GM   Colony-forming units granulocyte–macrophage

CFU-GEMM   Colony forming unit that generates myeloid cells.

DC   Direct Current

EDTA   Ethylenediaminetetraacetic acid

FACS   Fluorescence-activated cell sorting

FBC   Full blood count

FITC   Fluorescein isothiocyanate

FSC   Forward scatter

G-CSF   Granulocyte-colony stimulating factor

GM-CSF	Granulocyte monocyte-colony GM-CSF stimulating factor
HF	High Frequency
HFLC	High fluorescence lymphocyte count
HLA	Human leucocyte antigen
HPC	Haematopoietic Progenitor cells
HSCs	Haematopoietic stem cells
HUSM	Hospital Universiti Sains Malaysia (HUSM)
ICC	Intraclass correlation coefficient
IG	Immature Granulocytes
IMI	Immature information
IPF	Immature platelet fraction
IRF	Immature reticulocyte fraction
Kb	Kilobyte
LB	Lower bound
LFR	Low fluorescence reticulocyte
LSI	Left-shift index
mAb	Monoclonal antibody
MFR	Medium fluorescence reticulocyte
MSC	Marrow stem cells
NK	Natural killer
NRBC	Nucleated Red Blood Cells
PB	Peripheral blood
PBSC	Peripheral blood stem cells

PCR	Polymerase chain reaction
PE	Phycoerythrin
PLT-O	Platelets by optical fluorescence
rhG-CSF	recombinant human granulocyte colony-stimulating factor
RET-H*e*	Reticuloyte Hemoglobin
RNA	Ribonucleic acid
ROC	Receiver operating characteristics
RETIC	Reticulocytes
SC	Stem cells
SD	Standard deviation
SPSS	Statistical Packages for Social Sciences
UCB	Umbilical cord blood
UB	Upper bound
WBC	White blood cells
7-AAD	7-amino actinomycin D

7. ABSTRAK

PEMBANDINGAN BILANGAN SEL CD 34+ YANG DITENTUKAN OLEH MESIN ANALISIS HEMATOLOGI SYSMEX DAN "FLOW" SITOMETRI.

Antigen CD34+ berada di sel prekursor darah yang pramatang dan di semua koloni yang membentuk sel darah di dalam sum-sum tulang dan darah. Dengan memberi " recombinant human granulocyte colony-stimulating factor" (rhGCSF), ini adalah cara yang efisyen untuk mengalakkan sel stem dari sum-sum tulang untuk keluar ke sirkulasi darah periferi. Keputusan untuk menuai "peripheral blood stem cells" adalah berdasarkan pengiraan sel CD34+ yang diperolehi dari mesin "flow" sitometri yang memerlukan pemantauan bilangan CD34+ setiap hari selepas diberi regimen kemoterapi "mobilization regimens". Pada masa kini, pengiraan sel CD34+ akan dimulakan apabila bilangan sel darah putih lebih daripada 1 X 10^3/μL. Mesin "flow" sitometri adalah kaedah yang ditetapkan untuk mengira sel CD34+ sebelum pengumpulan "peripheral blood stem cells".

Walaubagaimanapun, "flow" sitometri adalah mesin yang menggunakan teknik yang mengambil masa yang lama, kompleks dan memerlukan kos yang tinggi. Baru-baru ini, teknologi baru telah menghasilkan mesin analisis hematologi rutin Sysmex yang membolehkan pengesanan "Haematopoietic Progenitor cells" (HPC). Pengesanan dan

pengiraan HPC oleh mesin analisis hematologi rutin Sysmex boleh dijadikan sebagai alternatif yang berkesan dari segi kos dan jangkamasa.

Objektif kajian ini adalah 1) untuk membandingkan bilangan HPC yang ditentukan oleh mesin analisis hematologi Sysmex dan bilangan sel CD34+ yang dikira oleh mesin flow sitometri ; 2) untuk membandingkan sel CD34+ yang dikira oleh mesin flow sitometri dengan bilangan sel darah putih (WBC), "immature platelet fraction" (IPF), "reticulocytes" (RETIC) dan "immature retic fraction" (IRF) yang ditentukan oleh mesin analisis hematologi Sysmex dan 3) untuk mengkaji sama ada mesin analisis hematologi Sysmex boleh meramal sel CD34+ seperti dikira oleh mesin "flow" sitometri.

Kajian ini telah dijalankan dari November 2008 hingga Jun 2009 ke atas pesakit kanser darah yang mendapat rawatan di Wad Hematologi, HUSM dan di kalangan bayi yang baru lahir di Wad Bersalin, HUSM. Sebanyak 68 sampel darah telah di ambil dari 19 orang pesakit kanser darah yang diberi rawatan kemoterapi dan GCSF serta 95 sampel darah diambil dari tali pusat bayi yang baru lahir. Sampel darah di atas dianalisa oleh mesin analisis hematologi Sysmex XE-2100 untuk menentukan bilangan HPC, WBC, IPF, RETIC and IRF dan oleh mesin flow sitometri untuk bilangan sel CD34+. Keputusan kajian dianalisa menggunakan program SPSS versi 12.

Analisis sampel darah tali pusat menunjukkan "mean(SD)" untuk bilangan HPC ialah 32.6 (34.3) X 10^6/L dan "mean(SD)" bilangan CD34+ ialah 36.8 (27.2) X 10^6/L. Keputusan

kajian ini menunjukan bahawa tiada perbezaan signifikan di antara "mean" bilangan HPC and "mean" bilangan sel CD34+.

Korrelasi Spearman bagi analisis sampel darah pesakit, kanser darah menunjukkan terdapat perhubungan signifikan di antara bilangan sel CD34+ dengan HPC $(r = 0.703$, $p<0.001)$, WBC ($r = 0.482$, $p<0.001)$, Retic ($r = 0.498$, $p<0.001)$ and IPF ($r = 0.453$, $p<0.001)$ tetapi tidak dengan IRF ($r = -0.073$, $p = 0.552)$.

Salah satu objektif kajian ini ialah untuk menentukan nilai yang optimum bagi parameter hematologi yang boleh meramal bilangan sel CD34+ yang > 20 x 10^6/L. Ini kerana pada masa ini, masa untuk proses menuai "peripheral blood stem cell" dimulakan adalah berdasarkan bilangan sel CD34+ > 20 x 10^6/L. Keputusan kajian ini ialah nilai HPC > 21.5 X 10^6/L dengan sensitiviti dan spesifisiti, 77% dan 64%, IPF > 26.1% dengan sensitiviti 77% and spesifisiti, 50%, RETIC > 1 X 10^9/L dan sensitiviti and spesifisiti adalah 73% and 64% , WBC > 23.1 X 10^9/L dengan sensitiviti and spesifisiti, 73% dan 50% dan IRF > 8.9% dengan sensitiviti and spesifisiti, 50% dan 52%.

Hasil kajian ini menunjukkan terdapat perhubungan signifikan di antara bilangan sel CD34+ yang diukur oleh "flow" sitometri dengan bilangan HPC, RETIC, WBC, IPF yang di kira oleh mesin analisis hematologi Sysmex XE-2100 tetapi tidak bagi IRF.

Secara kesimpulannya, hasil kajian ini menunjukkan bahawa bilangan HPC, RETIC, WBC, IPF boleh digunapakai ke dalam protokol klinikal untuk menentukan masa yang sesuai untuk "leukapheresis" bagi memulakan pengumpulan "peripheral blood stem cells".

8   ABSTRACT

A COMPARISON OF HAEMATOPOIETIC PROGENITOR CELLS ENUMERATION USING SYSMEX HEMATOLOGY ANALYZER AND STANDARD FLOW CYTOMETRY.

The CD34+ antigen is present on immature haematopoietic progenitor cells and all haematopoietic colony-forming cells in bone marrow and blood. The administration of recombinant human granulocyte colony-stimulating factor (rhG-CSF) has emerged as an efficient way to accomplish mobilization peripheral blood stem cells (PBSCs). The decision to harvest PBSC is based on the enumeration of CD 34^{+} cells by flow cytometry in which require daily CD34+ monitoring after given chemotherapy-based mobilization regimens. Currently, CD34+ enumeration will be initiated when the white blood cell (WBC) count is more than 1×10^{3} /µL. Flow cytometry assays have been used as a standard method for CD34+ enumeration in leukapheresis products before peripheral stem cell collection. However, flow cytometry techniques are time consuming, complex and expensive. Recently, technology has become available on a routine haematology analyzer that enables the detection of hematopoietic progenitor cells (HPC). Detection and enumeration of HPC by Sysmex haematology analyzer could provide a standard, cost effective and rapid alternative for predicting the yield of stem cells.

The aims of this study were 1) To compare Haematopoietic Progenitor cells (HPC) enumeration by using Sysmex haematology analyzer and CD34+ count by standard flow cytometry in cord blood. ; 2) To correlate the number of CD34$^+$ cells by standard flow cytometry with Haematopoietic Progenitor cells (HPC), white blood cells (WBC), immature platelet fraction (IPF), Reticulocyte (retic) and immature reticulocyte fraction (IRF) acquired by Sysmex haematology analyzer in patient on Granulocyte growth stimulating factor (G-CSF) and 3) to predict the level of CD34+ cells by using the haematological parameters derived from Sysmex haematology analyzer.

A cross sectional study was done in Stem Cell laboratory at HUSM from November 2008 to June 2009. 3mls of EDTA anticoagulated blood from cord blood of 95 newborns were collected and 68 samples of 3mls EDTA anticoagulated blood were collected from 19 adult patients with haematological malignancies from HUSM who had received chemotherapy-based mobilization regimens including G-CSF. Progenitor cell quantification was performed measuring HPC counts as well as other haematological parameters, WBC, IPF, RETIC and IRF counts provided by the Sysmex XE-2100 haematology analyzer and CD34$^+$ counts obtained in parallel by flow cytometry. Data were analyzed using the Statistical Packages for Social Sciences (SPSS) software version 12.0.

Analysis of the umbilical cord blood showed that mean(SD) for HPC count was 32.6 (34.3) X $10^6/$ L and mean(SD) for CD34+ count was 36.8 (27.2) X $10^6/$ L. There was no significant difference between mean HPC count and CD34+ count.

Blood samples from adult patient with haematological malignancies, were analysed by Spearman correlation, showed a highly significant correlation between CD34+ and HPC count (r = 0.703 , p<0.001), WBC (r = 0.482 , p<0.001), Retic (r = 0.498 , p<0.001) and IPF (r = 0.453 , p<0.001) but not with IRF (r = -0.073 , p = 0.552).

The other objective was to determined the optimal cut-off point for the haematological parameters that predicts CD34+ count of > 20 X $10^6/$ L. This is because in current practice the harvest will be initiated when CD34+ count is > 20 X $10^6/$ L. The result showed that HPC, IPF, Retic, WBC and IRF threshold were > 21.5 X $10^6/L$, > 26.1%, > 1% , > 23.1 X $10^9/L$ and > 8.9% respectively with sensitivity and specificity were (77% and 64%, 77% and 50%, 73% and 64% , 73% and 50% and 52% respectively).

In this study we found that, there is a significant correlation between the number of CD34^{+} cells measured using flow cytometry with HPC, RETIC, WBC, IPF measured by Sysmex haematology analyzer but not with IRF.

This study revealed that HPC, WBC, RETIC and IPF counts can be applied independently into clinical protocol to evaluate the timing of leukapheresis for starting PBSC collection.

Chapter 1

Introduction

1.0   GENERAL INTRODUCTION

Haematopoietic stem cells (HSCs) are multipotent stem cells and the most primitive of all blood cells that is capable of dividing and differentiating into specific blood cells type required by the body including myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid lineages (T-cells, B-cells, NK-cell) (Figure 1.1).

The definition of haematopoietic stem cells has undergone considerable revision in the last two decades. The haematopoietic tissue contains cells with long-term and short-term regeneration capacities and committed multipotent, oligopotent, and unipotent progenitors.

HSCs are found in the bone marrow of adults, which includes femurs, hip, ribs, sternum, and other bones. Other sources for clinical and scientific use include umbilical cord blood, placenta and mobilized peripheral blood.
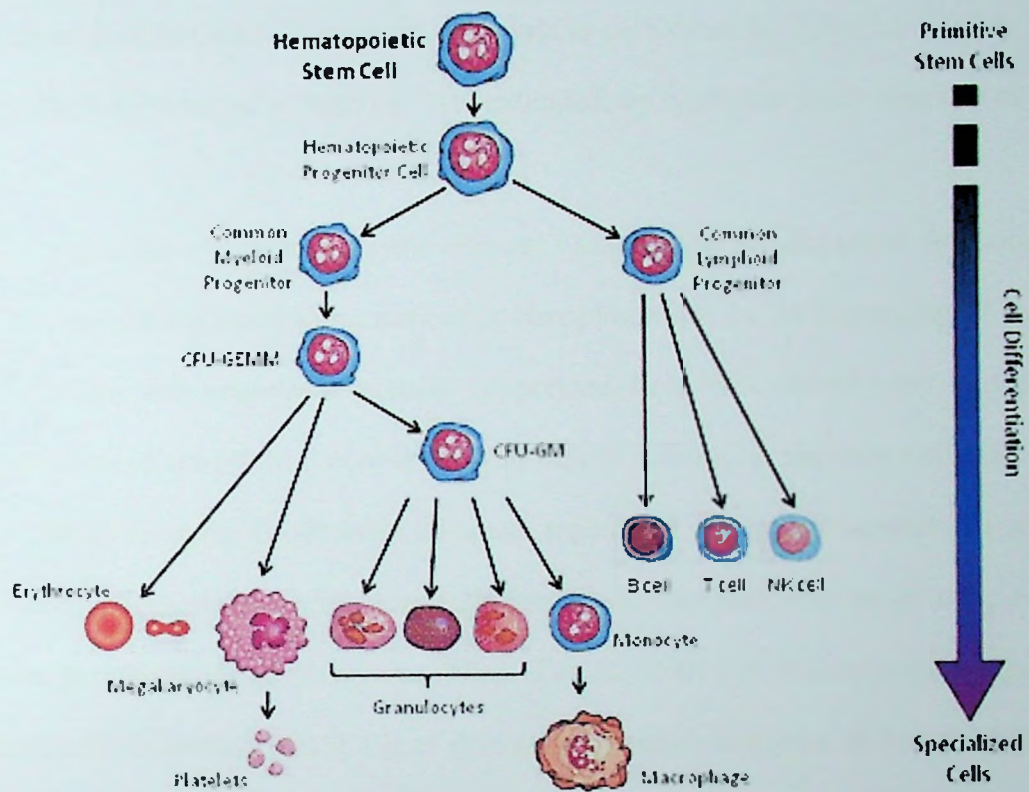
Figure 1.1   Haematopoietic Stem Cells differentiation.

(Adapted from ImmuneRegen Biosciences, Inc.)

Haematopoietic progenitor cells can be harvested from marrow as well as umbilical cord blood and from the blood following pre-treatment with cytokines, such as G-CSF (granulocyte colony-stimulating factors), that induce cells to be released from the bone marrow compartment. Stem cells transplant is performed to save the marrow of patient who are likely to be cured or their survival extended, by high dose chemotherapy or radiotherapy.

Nowadays, peripheral blood stem cells are harvested as an alternative for bone marrow cells in allogeneic haematopoietic stem cells transplantation. In the harvesting of peripheral blood stem cells, the marrow is usually suppressed first by chemotherapy and then G-CSF is administered around the recovery period before harvesting the stem cells (K. Kawakami *et al.*, 2006). In order to obtain an adequate amount of cells, it is necessary to approximate the number of peripheral blood stem cells harvested after mobilization utilizing results obtained from peripheral blood specimens (Nina G *et al.*, 2001). A crucial point for peripheral blood stem cells collection (PBSC) is to determine precisely the time of haematopoietic stem cells (HSCs) collection has to begin.

Expression of CD34+ is a defining hallmark of haemopoietic stem cells and progenitor cells and this marker is therefore widely used as a tag for the enumeration, isolation, and manipulation of HSCs (Healy *et al.*, 1995).

Haematopoietic progenitor cells, which is also identified by the expression of CD34+ surface antigen, are present in bone marrow (BM) and can be detected in steady state peripheral blood, though at a very low level. During the recovery phase from non-ablative chemotherapy administration, there is a well-documented increase in the number of CD34+ peripheral blood progenitor cells (PBPCs) that can be further enhanced by adding growth factors. These cells can be estimated by flow cytometry and their large-scale collection requires leukapheresis, often performed on consecutive days if their blood level remain high.

Cryopreserved for transplantation, PBPCs are able to engraft and to restore the haemopoiesis after myeloablative therapy. However, the rise of PBPCs is of short duration after the phase of mobilization. Thus, it is crucial to begin leukapheresis during this period, when an important overshoot of PBPCs appears. In addition, optimal timing and number of leukapheresis that need to be performed are essential in all patients to obtain a sufficient amount of PBPCs CD34+ for a safe autografting (Giovanni D'Arena et al., 1998).

The optimal point to initiate leukapheresis is estimated by the combination of leukocyte counts and CD34+ measurements. To this end, daily CD34+ tests have to be performed using flow cytometry, which are laborious and time consuming (R. Kraai et al., 2001). An alternative for this test might be the application of the "immature information" (IMI) channel measurement of the automated haematology analyzer Sysmex XE-2100 which more faster, easier and inexpensive.

1.1 Rationale of the study

In view of the clinical importance of CD34+ enumeration as a guide for harvesting of stem cells for an autologous stem cells transplantation. The knowledge and understanding of CD34+ cells and its association with others hematological parameters will assist us to predict the time for leukapheresis.

Currently, as a routine practice the decision to harvest PBSC is based on CD34+ cells enumeration by standard flow cytometry and need daily CD34+ monitoring until the optimal number is obtained. Conventionally, standard flow cytometer that uses a fluorescent antibody is generally used for counting CD34+ cells, which requires at least 2 hours even when done by an experienced technician and cost about RM 70.00 per test (as calculated based on test done at Haematology laboratory, HUSM).

Therefore, there is a need for much simpler and faster method to determine the suitable time for harvest. The IMI channel of Sysmex automated haematology analyzer is very useful for obtaining the HPC count because the procedure is very simple, non-tedious, faster which require only about 90 seconds for the analysis which should be a great advantages and sample for FBC can be used. It cost only RM 15.00 per sample (as calculated based on test done at Haematology laboratory, HUSM).

The results obtained from this study showed that the enumeration of HPC may be a valuable tool for laboratories and apheresis centers that have access to this haematology analyzer as a guide to determine when to initiate apheresis after chemotherapy-based mobilization regimens.

Chapter 2

Literature Review

2.0 LITERATURE REVIEW

2.1 Stem cells

2.1.1 Introduction

Stem cells have the remarkable potential to develop into many different cell types in the body during early life and growth. In addition, in many tissues they serve as a sort of internal repair system, dividing essentially without limit to replenish other cells as long as the person is still alive. When a stem cell divides, each new cell has the potential either to remain a stem cell or become another type of cell with a more specialized function, such as a muscle cell, a red blood cell, or a brain cell. Stem cells are distinguished from other cell types by two important characteristics. First, they are unspecialized cells capable of renewing themselves through cell division, sometimes after long periods of inactivity. Second, under certain physiologic or experimental conditions, they can be induced to become tissue- or organ-specific cells with special functions. In some organs, such as the gut and bone marrow, stem cells regularly divide to repair and replace worn out or damaged tissues. In other organs, however, such as the pancreas and the heart, stem cells only divide under special conditions (Stem cell basics , U.S. Department of Health and Human Services National Institutes of Health).

Until recently, scientists primarily worked with two kinds of stem cells from animals and humans: embryonic stem cells and non-embryonic "somatic" or "adult" stem cells. The history of research on adult stem cells began about 50 years ago. In the 1950s, researchers discovered that the bone marrow contains at least two kinds of stem cells. One population, called haematopoietic stem cells, forms all the types of blood cells in the body. A second population, called bone marrow stromal stem cells (also called mesenchymal stem cells, or skeletal stem cells by some) were discovered a few years later. These non-haematopoietic stem cells make up a small proportion of the stromal cell population in the bone marrow, and can generate bone, cartilage, fat, cells that support the formation of blood, and fibrous connective tissue (Figure 2.1) (Stem cell basics, U.S. Department of Health and Human Services National Institutes of Health).
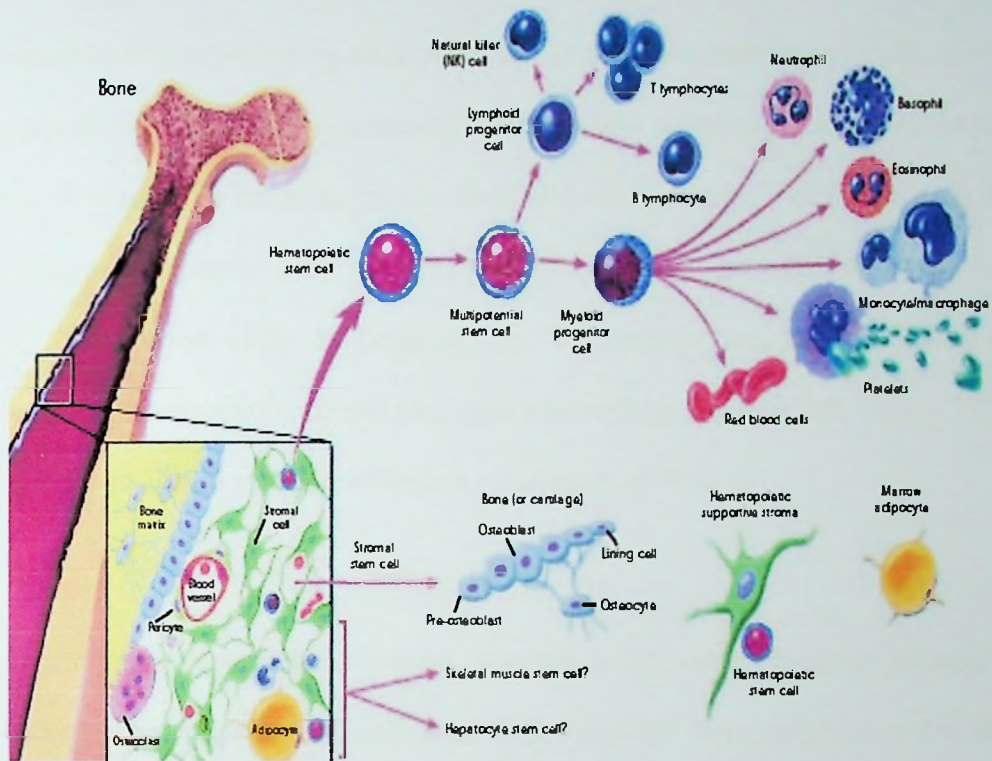
Figure 2.1   Hematopoietic and stromal stem cell differentiation.

Adapted from (Stem cell basics , U.S. Department of Health and Human Services

National Institutes of Health).

Adult stem cells typically generate the cell types of the tissue in which they reside. For example, a blood-forming adult stem cell in the bone marrow normally gives rise to the many types of blood cells. It is generally accepted that a blood-forming cell in the bone marrow— which is called a haematopoietic stem cell—cannot give rise to the cells of a very different tissue, such as nerve cells. Haematopoietic stem cells give rise to all the types of blood cells eg : red blood cells, B lymphocytes, T lymphocytes, natural killer cells, neutrophils, basophils, eosinophils, monocytes, and macrophages (Stem cell basics , U.S. Department of Health and Human Services National Institutes of Health).

Mesenchymal stem cell have been reported to be present in many tissues. Those from bone marrow (bone marrow stromal stem cells, skeletal stem cells) give rise to a variety of cell types: bone cells (osteoblasts and osteocytes), cartilage cells (chondrocytes), fat cells (adipocytes), and stromal cells that support blood formation. However, it is not yet clear how similar or dissimilar mesenchymal cells derived from non-bone marrow sources are to those from bone marrow stroma. (Stem cell basics , U.S. Department of Health and Human Services National Institutes of Health).

2.1.2 Identification of Stem cell

Stem cells can be identified by morphology, differentiation capacity and detection of antigen marker by flow cytometry. In order to identify stem cells and to characterize the differentiated cell types, stem cell "markers" have been introduced. Stem cell markers coating the surface of every cell in the body are specialized proteins, called receptors, that have the capability of selectively binding or adhering to other "signaling" molecules. There are many different types of receptors that differ in their structure and affinity for the signaling molecules. Normally, cells use these receptors and the molecules that bind to them as a way of communicating with other cells and to carry out their proper functions in the body. These same cell surface receptors are the stem cell markers. Each cell type, for example a liver cell, has a certain combination of receptors on their surface that makes them distinguishable from other kinds of cells. Scientists have taken advantage of the biological uniqueness of stem cell receptors and chemical properties of certain compounds to tag or "mark" cells (Bethesda., 2009)

Stem cell markers are given short-hand names based on the molecules that bind to the stem cell surface receptors. Stem cells are being identify by a combination of marker names reflecting the presence (+) or absence (-) of them.

The signaling molecules that selectively adhere to the receptors on the surface of the cell have been used as a tool that allows them to identify stem cells. A technique that has been developed was by attaching the signaling molecule to another molecule (or the tag) that has

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the ability to fluoresce or emit light energy when activated by an energy source such as an ultraviolet light or laser beam.

One approach is by using markers as a research tool. This technique is known as fluorescence-activated cell sorting (FACS). With this technique, a suspension of tagged cells (i.e., bound to the cell surface markers are fluorescent tags) (Figure 2.2) is sent under pressure through a very narrow nozzle—so narrow that cells must pass through one at a time. Upon exiting the nozzle, cells then pass, one-by-one, through a light source, usually a laser, and then through an electric field. The fluorescent cells become negatively charged, while nonfluorescent cells become positively charged. The charge difference allows stem cells to be separated from other cells. The researchers now have a population of cells that have all of the same marker characteristics, and with these cells they can conduct their research.
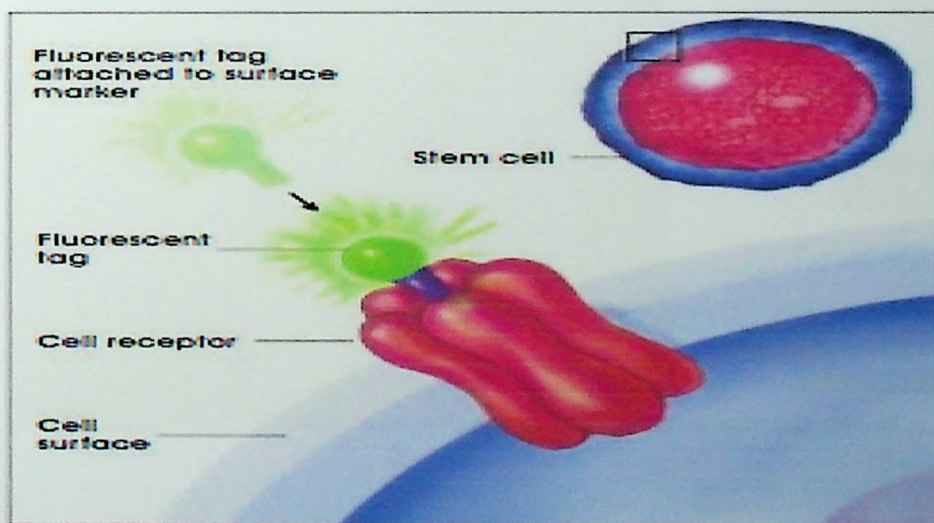
Figure 2.2   Identifying Cell Surface Markers Using Fluorescent Tags.

(Adapted from Bethesda., 2009)

Another method uses stem cell markers and their fluorescent tags to visually assess cells as they exist in tissues. Microscope are used to evaluate them rather than the FACS instrument. A thin slice of tissue is prepared, and the stem cell markers are tagged by the signaling molecule that has the fluorescent tag attached. The fluorescent tags are then activated either by special light energy or a chemical reaction. The stem cells will emit a fluorescent light and can easily be seen under the microscope (Figure 2.3).
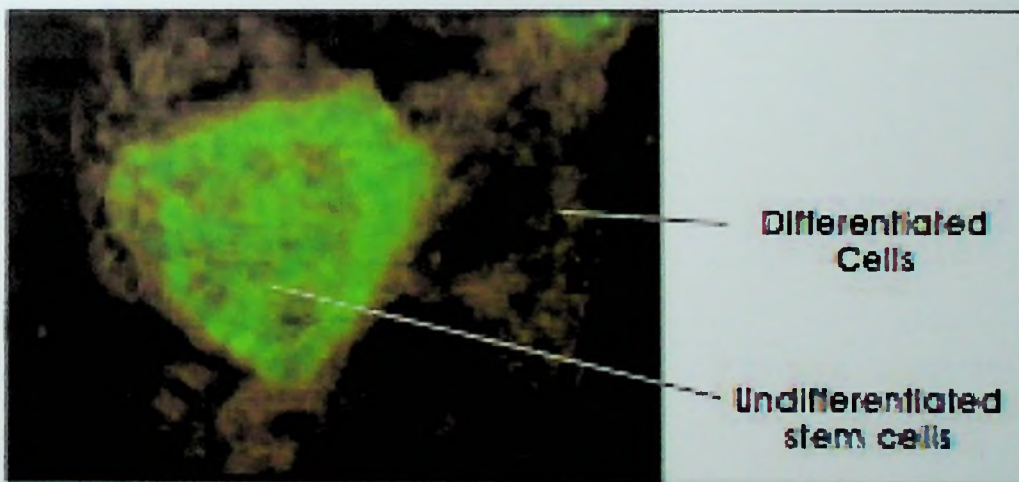
Figure 2.3   Microscopic Image of Fluorescent-Labeled Stem Cell.

(Adapted from Bethesda., 2009)

Genetic and molecular biology techniques are extensively used to identify the stem cells. Genes and transcription factors (proteins found within cells that regulate a gene's activity) are being identified. Techniques such as polymerase chain reaction (PCR) are used to detect the presence of genes that are "active" and play a role guiding the specialization of a cell.

2.2   CD34+ cells

2.2.1 Introduction

The CD34+ molecule is a membrane-associated glycoprotein present on precursors of both haematopoietic and stromal cells (R.A. Roscoe *et al.,* 1994). The CD34+ antigen is expressed by early haematopoietic stem cells and progenitors and is detected on the surface of approximately 1 % of bone marrow mononuclear cells (Robert H., 1993).

Expression of the stem cell antigen CD34+ is a defining hallmark of haemopoietic stem cells and progenitors and CD34+ is therefore widely used as a tag for the enumeration, isolation, and manipulation of these cells (Healy *et al.,* 1995). It is well established that the 3% of cells in the bone marrow express the CD34+ antigen. This cells are heavily glycosylated mucin-like structure and capable of reconstituting long term, multilineage haematopoiesis after myeloablative therapy (D. Barnett *et al.*, 1999).

Clinical applications of CD34+ cells include autologous transplantation of putative CD34+ stem cells isolated by positive selection from the bone marrow or peripheral blood stem cells, using the proportion and number of CD34+ cells as a guideline for the harvesting procedure (Silvestri *et al.,* 1992).

CD34+ cells are also found in the peripheral blood of normal individuals but are extremely rare (approximately 0.01–0.05% of total nucleated cells). Peripheral blood stem cells (PBSC) have now virtually replaced bone marrow as the primary source of stem cells for autologous transplantation and the procedure is being increasingly used for allogeneic transplantation between HLA-identical siblings (D. Barnett *et al.,* 1999).

Potential advantages of PBSC transplantation include a more rapid haematopoietic reconstitution, lower risk of contamination by tumour cells, reduced hospitalization, increased numbers of T-lymphocytes and NK cell that may reduce post-transplant relapse, and the elimination of a need for general anaesthesia (D. Barnett *et al.,* 1999).

Several monoclonal antibody-based methods have been developed to isolate these cells from clinical samples of bone marrow or peripheral blood based on their expression of this antigen, utilizing either biotin-avidin affinity, panning or immunomagnetic beads (Robert H., 1993).

2.2.2 CD34+ gene

Using somatic cell hybrids and in situ hybridization, the gene encoding CD34+ was localized to chromosome 1, band q32. Using the human cytoplasmic Deoxyribonucleic acid (cDNA) probe under conditions of lower stringency, the murine CD34+ cDNA has also been cloned and contains eight coding exons. The human gene, which covers about 26 Kb in length, is very similarly organized. The murine and human cDNAs are highly homologous: the cytoplasmic domains (encoded by exon 8) are >90% identical. The transmembrane and proximal extracellular regions (encoded by exons 6 and 7) and the cysteine-rich domain (encoded by exons 4 and 5) are also very similar (>75% and >70% identical respectively). The N-terminal domains of about 145 amino acids (encoded by exons 2 and 3) are only 45% sequence-identical. However, the presence of high levels of serine and threonine residues in this domain is conserved between the species, suggesting that the 0-linked carbohydrate moiety may determine the functional capabilities of this part of the CD34 + structure (Sutherland *et al.,* 1993).

Other posttranslational modifications such as glycosaminoglycan conjugation, phosphorylation by a variety of kinases, glycoprotein half-life, etc. of the CD34+ polypeptide may also modulate this structure's functional capabilities. Although the function remains elusive, the ability to use antibodies to the CD34+ antigen for the purification of stem progenitor cells for transplantation has some important implications for the intensive treatment of selected malignancies, as well as possibly providing potential target cells for genetic manipulation studies in vitro (Sutherland *et al.,* 1993).

"Positive selection" of hematopoietic stem progenitor cells represents an alternative strategy to purging by the "negative selection" of tumor from bone marrow prior to transplantation. In many tumor types treated with intensive therapy and stem cell rescue, malignant cells contaminating the marrow do not express CD34+, and tumor-free autografts may best be obtained by employing the "positive selection" approach afforded by selective CD34+ expression on haematopoietic stem cells (Sutherland *et al.*, 1993).

2.2.3   Function and Clinical applications

Haematopoietic stem cells have been widely used as part of the therapy for a variety of haematological and non-haematological malignancies. The source of haematopoietic cells can be autologous or allogeneic, collected from marrow, peripheral blood, or cord blood. Because of the low level of such cells in blood, growth factors, such as granulocyte-colony stimulating factor (G-CSF) and granulocyte monocyte-colony stimulating factor (GM-CSF), are administered to the patients (autologous) or healthy donors (allogeneic) to ''mobilize'' or increase the proportions of circulating stem cells. After several days of mobilization peripheral blood mononuclear cells are collected by leukapheresis. Expression of the CD34+ surface marker is commonly used to determine the stem cells count. The marker is expressed on primitive as well as committed stem cells, and the levels are 1–5% in bone marrow, 0.1 to 3% in cord blood, , 0.1% in non-mobilized peripheral blood, and 0.1 to 10% in mobilized blood. Most blood and marrow centers use number of CD34+ cells to determine the adequacy of the stem cell grafts (Ping Law *et al.*, 1999).

Cytometric analysis has become an important aspect in the quality control of cells in all phases of haematopoietic cell transplantation (Ping Law *et al.,* 1999). CD34+ enumeration by flow cytometry is used to decide when to collect PBSC, to assess HSC content in peripheral blood, BM and cord blood harvests and to predict the likelihood of engraftment or successfulness of stem cells infusion. CD34+ enumeration by flow cytometry is expensive, requires expertise and takes a minimum of 1-2 hours to perform (A. Padmanabhan *et al.,* 2009).

2.3   Reticulocytes

2.3.1   Definition

Reticulocytes are the erythroid cells in the peripheral blood that are in a discrete, penultimate phase of maturation. Then nucleus has been removed, usually before the red cells enter the peripheral blood. However, some of the extranuclear RNA remains. This residual RNA generally is lost progressively during the 24 hours after the cell enters the circulation. Reticulocytes differ from other red cells in that they have a more convoluted shape, and larger than the more mature cells. With the typical Wright's stain used for routine examination, only the earliest reticulocytes with the most residual RNA will be "polychromatophil" (more bluish than the mature erythrocytes) (Bessman., 1990).

Ultrastructural studies have shown that nuclear extrusion usually occurs outside the sinusoids of the bone marrow, and that newly formed reticulocytes usually pass through pre-existing gaps in the walls of these sinusoids by diapedesis. Thus, the mature reticulocyte has no nucleus but has a few mitochondria and ribosomes and its cytoplasm stains predominantly pink because of the high concentration of haemoglobin. The cytoplasm still has a greyish tint due to the presence of ribosomes. When stained supravitally, the ribosomes precipitate into basophilic granules or a reticulum. Reticulocytes continue to synthesize haemoglobin for 24–48 hours after release from the bone marrow. On average, these cells are about 20% larger than mature red cells, which are circular flat, biconcave discs with a mean diameter of 8.5 microns (Hoffbrand *et al.*, 2005)

Ribosomes have property of reacting with certain basic dyes such as azure B, brilliant cresyl blue, or New methylene blue to form a blue or purple precipitate of granules or filaments. This reaction takes place only in vitally stained unfixed preparations. Reticulocytes can be classified into four groups, ranging from the most immature reticulocytes, with a large clump of reticulin to the most mature, with few granules of reticulin (Figure 2.4) (S. Mitchell Lewis *et al.,* 2006).

Figure 2.4   Red cells stained supravitally with new methylene blue

(Adapted from Lichtman *et al.*, 2005)

2.4   Immature reticulocytes fraction

Reticulocyte quantitation has long been accepted as a useful tool for providing information regarding erythropoiesis. Over the years, methods were developed to classify reticulocyte maturation according to the amount of RNA that is present in the cells. Young reticulocytes have become a focal point of research with the development of reticulocyte analysis by flow cytometry (C. Kessler *et al.,* 1995).

Immature reticulocytes, also called shift or stress cells, normally constitute less than five percent (5%) of the total number of reticulocytes. They contain the greatest quantity of RNA, which nucleases degrade over a period of three days. Young reticulocytes are released in the peripheral blood circulation during periods of intense erythropoietic stimulation (C. Kessler *et al.*, 1995).

Reticulocyte counting technology is traced from microscopic counts through the various forms of automation. Accurate measurements of reticulocyte maturation are possible using multi-functional and dedicated flow cytometers using florescent and supervital dyes. Immature reticulocyte fraction (IRF) measurements are described by many as useful for clinical management of various haematopoietic conditions (C. Kessler *et al.*, 1995).