EFFECTS OF bFGF (BASIC FIBROBLAST GROWTH FACTOR) ON THE HAEMATOPOIETIC SEQUELAE THAT FOLLOW TRANSPLANTATION

Prepared by: Seretloane Japhtaline Makgoka, Masters of Science in Medicine Student in the Department of Haematology, University of Cape Town

Supervised by: Professor Nicolas Novitzky

Prepared for: The Department of Haematology at the University of Cape Town, Groote Schuur Hospital.

November 2000

Thesis Prepared in Fulfillment of the Requirements for the Degree of Masters of Science in Medicine (Haematology).

ACKNOWDGEMENTS

This study was fully supported by the **Medical Research Council (MRC) of South Africa.** I would like to take this opportunity of thanking the council for all the financial assistance.

I am more grateful to the following people:

- **Professor Nicolas Novitzky** for all the time he had given to me even under difficult situations, for the thorough discussions and for critically reading my manuscripts.
- **Dr Donavon Hiss** for thoroughly reading my manuscripts, all the good advices he had provided and for always comforting me.
- **Rygana Mohamed** for the excellent technical training she had provided.
- **Valda Thomas** for the helpful discussions and intellectual advice.
- **Glenda Davison** and **Sebastian Gelderbloem** for their assistance in the running of the flow-cytometry machine.
- **Medical School Haematology Laboratory staff (Huibriëtte, Riva, Sherae, Tina)** for just being there when I needed to talk and laugh.
- **Groote Schuur Haematology Laboratory staff** for the organizing and collecting bone marrow aspirate samples.
- **Coleen Fredericks and Wardah Jacobs** for conveying my messages from Prof. Novitzky on time. For helping me with the typing and editing of my important letters and documents.
- **Jean Aprill and Augustine Bhonga** for collecting my specimens from the hospital
- **Fatima Haupt** for her motherly care and important advices she gave me.
- **The Writing Center Staff Members** for carefully reading through my manuscripts

DEDICATIONS

This dissertation is dedicated to my late father, **Kgabo Moses**. I want to thank him for the love and fatherly advice he showed me before his death.

To my mother, **Mathabatha** for her undying love, financial assistance, patience, support and above all for giving me this wonderful opportunity of having had the chance to gain an excellent education.

It also goes to my brothers: **Chuene, Koena. J, Koena. C, Phuti, Tati, my sister Ditebogo, sisters in-law, my nephews, nieces** and last but not least my best friend, **Kgopotso** for always been there when I needed a shoulder to cry on. To **all my friends**, I just want to say that I did not finish this dissertation alone, meaning that it also belongs to you.

ABBREVIATIONS

ALL = Acute Lymphoblastic Leukaemia

AML = Acute Myeloid Leukaemia

bFGF = basic Fibroblast Growth Factor

BMT= Bone Marrow Transplantation

CFU-bl = Colony Forming Unit-blast

CFU-F = Colony Forming Unit-Fibroblast

CFU-GM = Colony Forming Unit-Granulocyte-Macrophage

CML = Chronic Myeloid Leukaemia

CSF-GM = Colony Stimulating Factor-Granulocyte-Macrophage

Cy = Cyclophosphamide

ECM = Extracellular Matrix

Epo = Erythropoietin

FCS = Foetal Calf Serum

HC = Horse Serum

HS = Heparan Sulphate

IL = Interleukin

MDS = Myelodysplastic Syndrome

MNC = Mononuclear Cells

MTx = Methotrexate

NHL = non-Hodgkin's Lymphoma

PBSC = Peripheral Blood Stem Cells

SAA = Severe Aplastic Anaemia

SCF = Stem Cell Factor

TBI = Total Body Irradiation

TGF-β = Transforming Growth Factor-β

TNI = Total Nodal Irradiation

α-MEM = α-Minimal Essential Medium

CONTENTS

Title **Page Number**

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

ILLUSTRATIONS

CHAPTER 1

Figure **Page Number**

CHAPTER 4

Picture **Page Number**

CHAPTER 5

ABSTRACT

Recovery following bone marrow transplantation is associated with the reduction in the clonogenic potential of stroma-adherent $CD34^+$ progenitors. The bone marrow stroma is also affected, resulting in poor support for the growth of stroma-adherent multipotent progenitors that form blastic colonies (CFU-bl). To determine the possible mechanisms of marrow damage in patients treated with peripheral blood stem cells (PBSCs) and bone marrow transplantation (BMT), we studied the effects of bFGF, a cytokine known to stimulate the survival and proliferation of fibroblasts, on the propagation of clonogenic progenitors and the marrow stroma.

METHODS: In order to obtain the optimal bFGF concentrations to be used on patients' haematopoietic progenitors and bone marrow microenvironment, haematologically normal individuals were studied in dose response studies. Control mononuclear cells from the Ficoll-Histopaque interface layer were divided into two aliquots: one to establish a stromal layer and to culture fibroblastic progenitors (CFU-Fs) in the presence of 0, 2 and 20 ng/ml bFGF with and without 20 ng/ml heparan sulphate (HS). The second aliquot was for the selection of the progenitor population. Stroma was quantitated by placing culture dishes on a grid containing 1mm squares and scoring the number of squares occupied by stromal layers as the percentage area covered. After 3 weeks of culture, a single cell suspension was prepared by incubation of stroma with 5% trypsin solution, and number of cells in the dishes enumerated with a particle counter. CFU-Fs were terminated on the 9^{th} day of culture, stained with May-Grünwald-Giemsa and scored using an inverted microscope.

From the second aliquot, $CD34^+$ cells were incubated with paramagnetic beads and target cells isolated with a magnet. Selected 1×10^4/ml cells were cultured on preformed controls' stroma that has been treated with 0, 2 and 20 ng/ml bFGF. Stroma-adhered cells were covered with 0.3% agar and cultured for 6 days. Aggregates of more than 20 cells were counted as CFU-bl.

RESULTS: In normal individuals, the median surface area of the petri dish covered by stroma at 3 weeks of culture was 55% (range 30-65) and was significantly improved upon the addition of 2 ng/ml (median 70%; range 50-95; $p = <0.05$) and 20 ng/ml bFGF (median 80%; range 65-99; $p = 0.004$). Stromal cell numbers were 0.61×10^6/2ml (range 0.15-1.66), and they increased significantly with the addition of 2 and 20 ng/ml bFGF ($p=0.03$). The median colony forming unit-blasts (CFU-bl) scores were 121.8×10^4/ml (range 43-271), and they expanded significantly with the addition of 20 ng/ml bFGF with and without heparan sulphate ($p=0.03$ and 0.01). It was then concluded that the 2 and 20 ng/ml bFGF with heparan sulphate be used on patients' cells in vitro.

In order to define the effects of the 2 and 20 ng/ml bFGF on the haematopoietic progenitors and on the bone marrow microenvironment, 16 patients in remission from their haematological malignancies, who had received grafts, were studied employing the same methods used for the normal population.

The median surface area covered by stromal layers in patients' samples at 3 weeks was 40% (range 0-55 vs. normal 50%), and it was improved significantly upon the addition of bFGF (median 78 vs. 50%; p= 0.001). Supplementation of stromal layers with bFGF accounted for a significant increase in patients stromal cell numbers (2.11 vs. normal 0.61 x 10^6/2ml; p< 0.05). Patients' $CD34^+$ cells panned on normal stromal layers resulted in significantly fewer CFU-bl (median 40 vs. normal 90 x 10^4/ml CFU-bl; p=0.009), but CFU-bl numbers were corrected following the addition of bFGF, matching the scores achieved by normal individuals (85 vs. normal 90 x 10^4/ml). Normal $CD34^+$ cells proliferated poorly on patients' stromal layers in the absence of bFGF (39 vs. normal 90 x 10^4/ml), but colony numbers increased significantly upon addition of bFGF (91 vs. normal 90 x 10^4/ml CFU-bl).

Thirteen patients receiving peripheral blood stem cells (PBSCs) and nine patients undergoing bone marrow transplantation (BMT) were compared. These two stem cell sources were then compared to the normal population. Stromal layers in patients receiving PBSC grafts covered a greater surface area than the area covered in patients undergoing BMT (median 65 vs. 30%). They also had higher blastic colony than scores from BMT recipients (47 vs. 12 x 10^4/ml CFU-bl; p= 0.2). Results can be summarized that bone marrow stroma from patients receiving mobilized progenitor cells proliferates better than those receiving bone marrow grafts.

It can be concluded that poor stromal layer and $CD34^+$ cell proliferation following peripheral blood stem cell transplantation can be corrected by addition of basic Fibroblastic Growth Factor.

CHAPTER 1

Haematopoiesis is the process whereby cells of both myeloid and lymphoid lineages differentiate from a common stem cell into mature haematopoietic cells [8,10]. It is characterized by the balanced interplay between haematopoietic progenitor cells, as well as molecules and cells that form part of the microenvironment. Cell adhesion molecules (CAMs) expressed on the cell surfaces of the haematopoietic progenitor cells bind to specific ligands on the stromal cells and the extracellular matrix (ECM) components. This interaction is important in that haematopoietic cells will be in contact with the growth factors produced by the stromal components. Heparan sulphate proteoglycans (HSPGs) are components of the extracellular matrix produced by stroma. They also play an important role in haematopoiesis because they bind to growth factors and present them to the haematopoietic cells within the microenvironment in an active form. The haematopoietic microenvironment is a highly organized structure where stem cells interact closely with the marrow stroma, and any alterations in the microenvironment may result in abnormal production of blood cells.

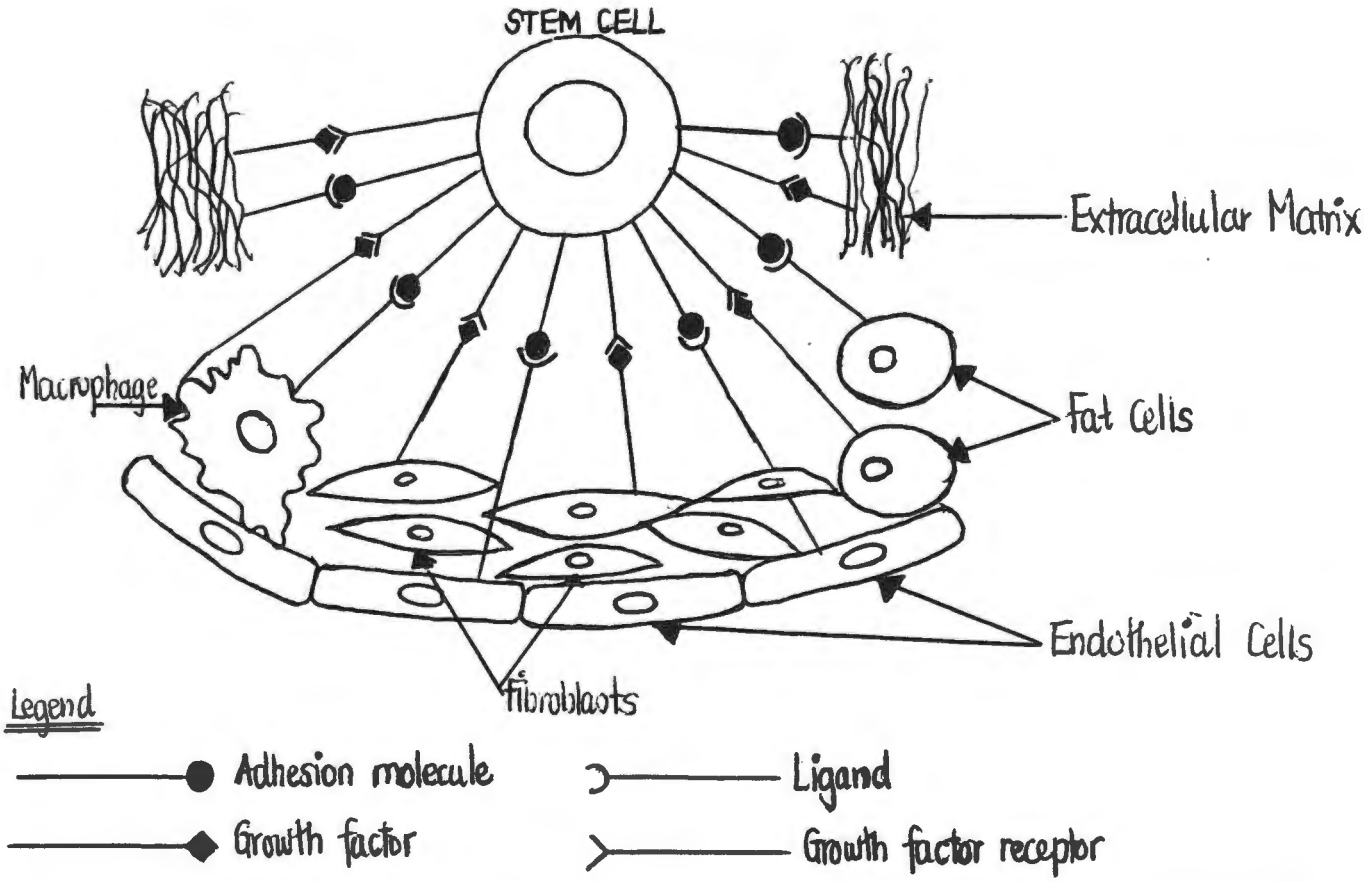


Figure 1. *Schematic diagram of the haematopoietic microenvironment. Stem cells express cell adhesion molecules, which bind to their specific ligands in the extracellular matrix. Growth factors are produced by stromal cells and they bind to their specific receptors on the stem cells.*

1.1 Introduction

The turnover of cells of the haematopoietic system in a man weighing 70 kg is estimated to be 1 trillion cells per day, and this includes 200 billion erythrocytes and 70 billion neutrophilic leukocytes [74,105].

In steady-state haematopoiesis, most progenitor cells reside in the bone marrow cavity and only a small percentage circulate in the peripheral blood. In the embryo, haematopoiesis starts between day 15 and 18 in the blood island of the yolk sac (a location that supports only erythropoiesis). After 6 weeks, haematopoietic stem cell migrates via the blood stream to the liver and the spleen where myeloid differentiation begins [5,95]. During the 20^{th} week of gestation, bone marrow haematopoiesis begins to occur, finally representing the whole system of blood cell production [98].

Within the bone marrow microenvironment, differentiation involves the haematopoietic stem cell, followed by long-term culture-initiating cell (LTC-IC), then colony forming unit-blast (CFU-bl) giving rise to the mixed colony forming unit-granulocyte-erythoid-macrophage-monocyte (CFU-GEMM) and finally the single lineage progenitors e.g., red cells, platelets, granulocytes (neutrophills, eosinophills), monocytes, and basophills.

1.2 Haematopoietic Stem Cells

Haematopoietic stem cells have three features: (1) a self-renewal capacity that is able to maintain a long-term supply of progenies, (2) a multilineage differentiation potential with the ability to clonally give rise to at least eight distinct cell lineages, (3) a proliferative potential that is responsible for the production of large numbers of mature blood cells.

Stem cells are recognized by the expression of a glycoprotein called the cluster of differentiation (CD) 34^{+} molecules. Clusters of differentiation (CD) are well-defined antigens that are recognized by specific monoclonal antibodies. This glycoprotein is expressed on the stem cell surface membranes; immature progenitor cells, as well as endothelial and fibroblastic progenitors. The $CD34^{+}$ expression disappears as cells differentiate into late progenitors and mature blood cells [47,48,67,82].

Cells that are included within the $CD34^+$ population can further be defined by co-expression of additional markers that can discriminate between the primitive stem cells and lineage committed haematopoietic progenitor cells [69,95], e.g. primitive (non-committed) stem cells express $CD34^+CD38^-$ while lineage committed cells express $CD34^+CD38^+$ [36,92].

The $CD34^+$ cell population comprises 1-3% of bone marrow mononuclear cells, and 0.01-0.1% of peripheral blood mononuclear cells. This $CD34^+$ cell population contains precursors for all lymphohaematopoietic lineages as shown by the finding that $CD34^+$ cells purified from marrow can reconstitute haematopoiesis in humans [2,5,41,48,49].

The $CD34^+$ selection is employed as a means to enrich stem cells in clinical transplantation protocols for multilineage engraftment and self-renewal [139]. The number of stem cells in $CD34^+$ lineage negative (Lin^-) fraction is 100-fold higher than the number of stem cells in $CD34^-$ Lin^- fraction, so it is preferred to engraft $CD34^+$ stem cells rather than $CD34^-$ cells [1,30]

Higher $CD34^+$ cell yields are obtained after positive selection from bone marrow aspiration performed during the day as opposed to late at night. It has been speculated that the temporal differences in physical properties related to cellular adhesiveness and distribution in the bone marrow space may be contributing factors. The $CD34^+$ cells are used in gene therapy studies and as markers for early haematopoietic stem/progenitor cells [49].

1.3 Long-Term Culture-Initiating Cell

The cell population that sustains long-term haematopoiesis when placed in a culture is called long-term culture initiating cell (LTC-IC). It represents a very primitive haematopoietic progenitor population, since it initiates and sustains committed myeloid progenitor growth for up to 12 weeks in long-term bone marrow cultures in humans. [29,54].

1.4 Colony Forming Unit-blasts

The human marrow also contains a population of progenitor cells called colony-forming unit-blast (CFU-bl). They are self-renewing cells that occupy an early position in haematopoiesis.

The CFU-bl is assigned a new name, viz., pre-progenitor cells, (PPC) which represent a population of transit cells and precede the lineage-committed progenitor cells [37,63]. The relationship between LTC-IC and CFU-bl is that CFU-bl is a descendant of LTC-IC.

1.4.1 Kinetics of Binding

CFU-bl can be distinguished by their ability to bind to preformed stromal layers and to form blastic colonies in the absence of magnesium, calcium, and serum, suggesting that the stromal layer is responsible for the requirements of binding. This interaction does not appear to involve known cell adhesion molecules (CAMs), neither prevented by the presence of monoclonal antibodies reacting with known phosphatidylinositol (PI)-linked structures, nor affected during trypsinization [31,37,38].

Binding of progenitor cells to stromal layers is mediated by heparan sulphate proteoglycans secreted as part of the extracellular matrix by stromal cells. Adhered progenitors produce colonies of undifferentiated blast cells, which are a uniform population of cells with a high nuclear: cytoplasmic ratio, no cytoplasmic granules, and little sign of maturation [31,37].

1.5 Long-Term Bone Marrow Cultures

Dexter and co-workers [61,76] first described long-term bone marrow cultures (LTBMC) in 1977. LTBMC reproduce the bone marrow microenvironment in vitro, thus allowing the identification of different components of the microenvironment and investigating their roles in haematopoiesis [88]. LTBMC can also be used to measure the combined injury to the haematopoietic stem cell and stromal cells and may reveal defects not apparent in short-term in vitro clonal assays. Colony forming unit-granulocyte macrophage (CFU-GM) assays are employed to demonstrate that the proliferative capacity and differentiation have been retained in LTBMC [22,53,61].

When bone marrow cells are cultured in vitro, an adherent cell layer referred to as a stromal layer must first develop before haematopoietic cells are generated [22,53]. This layer is produced when foetal calf serum, horse serum, and hydrocortisone are added to the initial culture medium. However, when only foetal calf serum is added to the culture medium, only a fibroblastic layer is formed [19,79,98].

The significant decrease in the total adherent cell number between weeks 8 and 9 is due to a significant decrease in fibroblasts, fat-containing cells and macrophages. The reason for this is that in older cultures, stromal layers form more three-dimensional bands, leading to a reduced cell contact to the glass surface and a greater tendency to become non-adherent [88].

1.6 Haematopoietic Microenvironment

The haematopoietic microenvironment in adult humans serves as a site of normal haematopoiesis. It consists of the haematopoietic cells, stromal cell layer (comprising endothelial cells, fat cells, fibroblasts, and macrophages), extracellular matrix (ECM), and growth factors. Haematopoietic cells seed within a stromal layer where they differentiate before being released into the culture supernatant as non-adherent cells [90]. If these haematopoietic cells were to be separated from stromal cells using a diffusion chamber on a layer of thin agar, they would die rapidly, implying a necessary intimate association with the stromal cells [22].

The heterogeneity of stromal cells within the LTBMC has complicated attempts to define the contribution of each component of the microenvironment in the regulation of haematopoiesis. Monoclonal antibodies (MoABs) are then used to provide a means of separating, characterising, identifying, and studying specific components in the heterogeneous system [77,80,88,90].

1.6.1 Stromal Layer Components

A. Endothelial Cells

Endothelial cells account for 10-20% of the adherent layer [3]. They form the morphologic and functional barriers between the vascular and haematopoietic compartments, where they exert controls over the cellular traffic between these two compartments, retaining the immature cells within the bone marrow [57]. The adhesive interactions between leukocytes and endothelial cells are central to homing of leukocytes to gastrointestinal tract and other lymph nodes, and are also likely to play a role in homing of haematopoietic cells [19,94].

B. Fat Cells

Fat cells occupy 5-10% of the adherent layer and are identified by their size, small nucleus, vacuolar cytoplasm and their positivity for the lipid-specific dye (Oil-O-Red). The appearance of fat cells in LTBMC depends upon the addition of hydrocortisone (HC) to the culture media [1] and they typically develop within four weeks in an adherent layer. The number of fat cells is increased with time in culture and this increase is associated with enlargement of their globules [3,19].

C. Fibroblasts

The fibroblasts represent a major component (60-70%) of the adherent layer. They are elongated, non-phagocytic, self-replicating cells known to be plastic adherent. The clonogenic progenitors responsible for fibroblastic colony formation are called colony forming unit- fibroblasts (CFU-Fs) [19,94].

Fibroblasts have a regulatory role in the differentiation and proliferation of haematopoietic cells in vitro, by producing paracrine and autocrine growth factors that can affect their own replication as well as that of nearby macrophages, endothelial cells and other cells of the bone marrow [19,40].

D. Macrophages

Macrophages account for 10-20% of the adherent cells of the human bone marrow stroma, and 70-90% of the murine bone marrow stroma. They play an important role in the regulation of haematopoiesis by producing cytokines such as IL-1α, IL-1β and GM-CSF [3]. These growth factors interact with haematopoietic cells directly or through specific cell surface receptors, which play a central role in the regulation of flow of haematopoietic progenitors into a required cell compartment [19,36,94].

The above-mentioned stromal layer secretes ECM components that are organized into a fibrillar network, surrounding the haematopoietic cells. The ECM components include collagen, fibronectin, haemonectin, heparan sulphate proteoglycans (HSPGs) and laminin, [3,19,94].

1.6.2 Extracellular Matrix Components

Fibronectin is a 450 kDa glycoprotein with a disulphide bond near the carboxy terminus. This molecule is responsible for mediating cell attachments, adhesion to collagen and laminin. The latter is a 900 kDa glycoprotein with three chains arranged in a cross-shaped tertiary structure, stabilized by disulphide bonds. Haemonectin is a 60 kDa glycoprotein that has only been identified in the marrow and believed to bind maturing granulocytes [20].

Heparan sulphate proteoglycans (HSPGs) are high molecular weight molecules in which sulphated glycosaminoglycans (GAGs) are covalently attached to a core protein [19,94]. GAGs are important components of the ECM and are also important in the regulation of haematopoiesis by binding and presenting cytokines to the haematopoietic cells in a more highly active form [87,96].

Heparan sulphates (HS) associated with bone marrow stromal cells participate in the adhesion of primitive haematopoietic cells to stroma, but functionally they vary due to differences in chemical structure [3,16,87]. Heparan sulphates are required for the maintenance of LTC-IC, while heparin, (a highly sulphated version of HS) directly activates growth factor receptor [96].

There are two classes of molecules that contribute to the maintenance of haematopoiesis: the growth factors, which have a well-documented capacity to stimulate survival, growth and development of primitive stem cells in vitro and cell adhesion molecules, which support the physical association of cells with the haematopoietic microenvironment.

1.7 Growth Factors

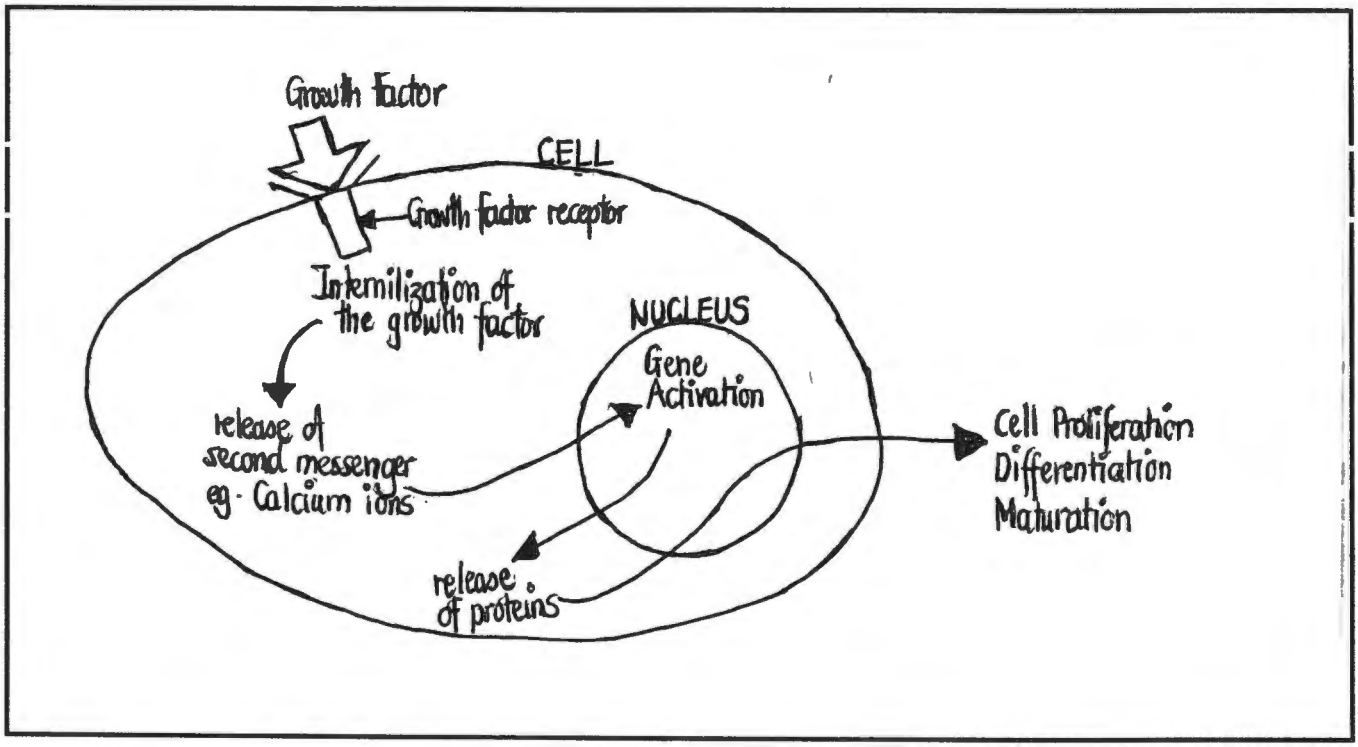


Figure 1. *Schematic view of the action of growth factors on the haematopoietic cells. Binding of growth factor to its specific receptor on the cell surface causes alterations in the intracellular domain of the receptor. This leads to the release of specific second messengers by activating one or other specific enzymes in the signal transduction pathway.*

Growth factors are a group of proteins that have potent biological activities, and are released by many cell types: the stromal cells, cells that make up the immune system, thymus, spleen, lymph nodes and other lymph organs. Growth factors are released at the site of tissue damage and participate in the reconstruction of cellular organization following an infection. Activation of cells is mediated by the interaction of growth factors with specific receptors expressed on the cell membrane surfaces, which then trigger intracellular events leading to the cytokine action [99].

The haematopoietic growth factor receptors belong to two major groups of transmembrane proteins: the tyrosine kinase receptor and the haemapoietin receptor family. The tyrosine kinase receptors have an extracellular ligand-binding domain with one or more protein molecules including immunoglobulin (Ig)-like, fibronectin type III and epidermal growth factor like domains.

This family includes stem cell factor (SCF), which has little colony-forming effect when used alone, but stimulates the *in vitro* proliferation of both myeloid and lymphoid haematopoietic progenitors when combined with other cytokines like erythropoeitin (Epo), interleukin-3 (IL-3), and granulocyte-macrophage colony stimulating factor (GM-CSF) [34,68].

Growth factors can be categorized into three main groups: (1) growth factors such as IL-1 and IL-2, which influence the growth and differentiation of B- or T-lymphocytes or their precursors; (2) growth factors such as IL-3, IL-6, SCF, which directly stimulate multipotent cells in terms of proliferation and development of colonies in vitro; (3) the colony stimulating factors (CSFs) such as GM-, G-, M-CSF, which influence the growth and development of myeloid progenitor cells. All these groups can further be divided into effector categories, e.g., survival, proliferation, and differentiation [26].

1.7.1 Cell Survival

Bone marrow cells removed from the body will only survive if they are cultured in the presence of growth factors. Haematopoietic cells depending on a specific growth factor for their survival need to be in the proximity of a stromal cell or matrix environment that can produce that growth factor or require the external addition of that specific growth factor into the culture medium. Cells outside the vicinity of such an environment will undergo a process of apoptosis. If the appropriate growth factor is present, they will survive and if the concentration of the growth factor is effective, they will proliferate and differentiate to produce more mature end cells [27].

1.7.2 Cell Differentiation

An initial phase when haematopoietic progenitor cells respond to an appropriate stimulus and develop into mature committed cells is called differentiation. Growth factors stimulate the stem cells to undergo differentiation, producing more developmentally restricted cells.

1.7.3 Cell Proliferation

The process when haematopoietic cells increase in number is called proliferation. Survival and proliferation are linked to the concentration of growth factors and the number of receptors they occupied at a given time.

This is demonstrated by the observation that when haematopoietic cells are cultured in media containing low concentration of a growth factor, they survive but do not proliferate. The cells will only start proliferating when the growth factor concentration is increased [27].

1.8 Basic Fibroblast Growth Factors

Fibroblast growth factors (FGFs), epidermal growth factor (EGF), and transforming growth factor-β (TGF-β) are produced by the stroma. FGFs belong to a complex family of mostly mitogenic proteins that share varying degrees of amino acid homology, but bind different receptors with variable affinity and gain extracellular exposure via different processes. They include factors like acidic (FGF-1), basic (FGF-2) and keratinocyte growth factor (KGF or FGF-7). A basic type of bFGF, which is used in this study has an isoelectric point (pI) of greater than 9, a molecular weight of 18500 Dalton [33,75,79].

1.8.1 Sources of bFGF

Basic FGF is isolated from bone, kidney, placenta, prostate, retina, brain, pituitary gland, and adrenal gland. It is synthesized by cultured normal and malignant cells and produced by human bone marrow cells. Both platelets and megakaryocytes might serve as its source in the peripheral blood and bone marrow [18,34,76].

1.8.2. Location of bFGF

Intracellular bFGF is localized in the cytoplasm, nucleus and nucleoli of many cell types while extracellular bFGF is deposited in bone marrow extracellular matrix as a complex with heparan sulphate proteoglycan (HSPGs) [15,17]. Basic FGF associated with HS proteoglycan constitutes a reservoir for this growth factor, protecting it from proteolytic degradation and therefore allowing it to mediate long-term biologic effects [3,33,103].

These bFGF-HS proteoglycan complexes were thought to be released in a biologically active form by incubation with exogenous inositol specific phospholipase C (PLC) or serine protease (plasmin) [15-17,40]. It was later noted that GPI-bFGF complexes are released from bone marrow cultures by endogenous glycophosphatidylinositol-phospholipase D (GPI-PLD) [16].

The mechanism of the release is thought to involve GPI-PLD, but not PLC because the released variant surface glycoprotein did not reveal an epitope called the cross-reacting determinant, which is exposed by PLC-catalyzed GPI anchor cleavage.

Glycosylphosphotidylinositol phospholipase D (GPI-PLD) not only regulates action of haematopoietic growth factors, but also mediate the cell-cell interactions in the bone marrow microenvironment [16].

1.8.2. Functions of bFGF

Basic FGF is a multifunctional growth factor that is involved in angiogenesis, embryogenesis, wound repair, and also haematopoiesis [15,35]. It accelerates the formation of a primary stromal layer following inoculation of newly harvested bone marrow cells into dishes. It is mitogenic for bone marrow stromal cells, because they attain high densities, lose their contact inhibition and grow in multilayered sheets in its presence [15,17].

Low concentrations of bFGF enhance myelopoiesis in LTBMC and result in an increase in the number of non-adherent and adherent layer derived progenitor cells [42]. Basic FGF also affects human megakaryocytes by directly promoting megakaryocytic progenitor cell proliferation and stimulating bone marrow accessory cells to release growth factors with the megakaryocytic colony stimulating activity, such as IL-3 and GM-CSF [17,34].

It further augments the proliferation of progenitor cells when added in conjunction with other growth factors and counteracts the suppressive effects of transforming growth factor (TGF)-β [64]. Basic FGF and SCF are synergistic in regulating stem cell proliferation and differentiation in the bone marrow [16,34].

1.9. Cell Adhesion Molecules

The second classes of molecules that contribute to the maintenance of haematopoiesis are cell adhesion molecules (CAMs). They are present on the surface membranes of progenitor cells and bind to their ligands on the stromal cells or the extracellular matrix components.

Restriction of early haematopoietic cell development and haematopoietic tissue involves expression of these CAMs [5,24,25,39]. Cell adhesion molecules belong to a six-structural family: integrins, selectins, immunoglobulin (Ig)-like molecules, CD44 molecule, cadherins, and sialomucin. Integrins are a large group of CAMs and are given this name because of their ability to integrate the intracellular cytoskeleton with extracellular matrix. Integrins are involved in cell migration and tissue organization during differentiation and inflammation, respectively, and mediate cell-cell and cell-ECM interactions [89].

Specific adhesion receptors on progenitor cells that mediate cell-ECM interaction have been described, viz., β_1-integrin adhesion molecule, very late antigen-4 (VLA-4), VLA-5 and VLA-6 and the receptor for hyaluronic acid (HA), CD44. Receptors like CD31/platelet endothelial-cell adhesion molecule-1 (PECAM-1), the β_2-integrin intracellular adhesion molecule-1, and also VLA-4 mediate cell-cell interactions [5,24,25,94].

The second groups of CAMs are selectins, which are divided into the L- and the P-selectins. The L-selectins are expressed on mature myeloid cells (e.g., neutrophils, eosinophils and monocytes) whilst a proportion of bone marrow endothelial cells express the P-selectins. Both selectins are involved in leukocyte adhesion to vascular epithelium and platelets [16,89].

The third group of CAMs are immunoglobulin (Ig)-like molecules that are expressed by human haematopoietic progenitor cells including LFA-3 (CD58), ICAM-1 (CD54) and Thy-1, while ICAM-1 and Thy-1 are also expressed by marrow stromal cells [100].

The fourth groups of CAMs are CD44, which are major cell surface receptors for hyaluronic acid and exhibit multiple activation states. Anti-CD44 antibodies identify primitive human haematopoietic progenitor cells and marrow stromal cells [100].

Interactions between progenitor cells, stromal cells and extracellular matrix (ECM) organize the cells to be in contact with growth factors needed for their proliferation and differentiation. Interaction of progenitor cells with endothelial cells is important for migration and homing of progenitors cells to the bone marrow. This involves tethering and rolling of haematopoietic progenitor cells along the endothelium.

Growth factors located on the endothelial surface or bound to proteoglycans in the endothelial extracellular matrix, activate the β_1 and β_2-integrins on the rolling haematopoietic progenitor cells, leading to firm adhesion to Ig-like receptors on the endothelial cells. This process is then followed by the transendothelial migration and anchoring of the progenitor cells in the bone marrow microenvironment [20,24,89].

1.10. Haematological Malignancies

Patients who participated in this study presented with the following haematological malignancies: chronic myeloid leukaemia (CML), acute myeloid leukaemia (AML), myelodysplastic syndromes (MDS), non-Hodgkin's lymphoma (NHL), acute lymphoblastic leukaemia (ALL), and severe aplastic anaemia (SAA).

1.10.1. Leukaemia

Leukaemia is a haematological clonal disorder, which is characterized by the uncontrolled proliferation of the haematopoietic progenitor cells, leading to accumulation of immature cells in the bone marrow or peripheral blood. Myeloid type of leukaemia arises from a transformation of haematopoietic stem cells accompanied by a reduced normal haematopoiesis. This change is brought about by the release of cytokines such as TNF-α from leukaemia cells [59,61]. Stromal compartments in leukaemia are not derived from the malignant clone and are sensitive to and traumatized by cryopreservation [59,69]. There are two types of myeloid leukaemia: the chronic myeloid leukaemia and acute myeloid leukaemia.

A. Acute Myeloid Leukaemia

Acute myeloid leukaemia (AML) is a malignant clonal disorder characterized by expansion of immature myeloid cells that are unable to differentiate fully, leading to accumulation of immature cells in the marrow or the blood. If it is left untreated, AML is more severe than CML because with AML, the survival period from diagnosis is only a few months, while with CML the period lasts for up to four years [50,59].

B. Chronic Myeloid Leukaemia

Chronic myeloid leukaemia (CML) is characterized by a clone that have the Philadelphia (Ph) chromosome, which results from the reciprocal translocation between chromosomes 9 and 22. This translocation leads to a fusion of the BCR and the c-ABL genes and the creation of a functional oncogene. CML develops in two phases: An initial chronic phase, which typically extends for years and a later blastic crisis, which is usually untreatable and rapidly fatal. Leukaemia cells present in patients with chronic phase CML differentiate normally. Other feature of CML is the presumed origin of leukaemia cells in a stem cell with lymphoid as well as myeloid differentiation potential [32,59].

1.10.2 Myelodysplastic Syndromes

Myelodysplastic syndromes (MDS) are characterized by the ineffective proliferation of cells from an abnormal stem cell leading to abnormalities of maturation and function of blood cells.

In the French-American-British (FAB) classification, they are categorized into refractory anaemia (RA), RA with ringed sideroblasts (RARS), RA with excess blasts (RAEB), RAEB in transformation (RAEB-t), and chronic myelomonocytic leukaemia (CMMoL). About 20-40% of MDS transform into acute leukaemia phase (post MDS AML) [56,65].

1.11 Bone Marrow Transplantation

Bone marrow transplantation (BMT) involves the elimination of an individual's bone marrow stem cells and all the cells derived from them including haematopoietic, lymphoid and macrophage systems. The eliminated cells are replaced with bone marrow stem cells either from another individual or with a previously harvested portion of the individual's own marrow. Since its introduction in the early 1970s, BMT has gained wide acceptance [21,72] and can either be allogeneic or autologous transplantation.

1.11.1 Allogeneic BMT

Allogeneic BMT is when bone marrow is transferred between siblings or HLA-matched individuals and is an effective therapeutic modality for the management of CML. Allogeneic BMT is associated with a high incidence of graft versus host disease (GVHD), which is mediated by T-lymphocytes.

Relapse of diseases after allogeneic BMT remains a major cause of treatment failure with a very poor prognosis. Minority of relapsed patients is cured with a second BMT, which results in significant morbidity and poor survival rates [19].

1.11.2 Autologous BMT

Autologous BMT is when bone marrow is collected from one individual and then reinfused after myeloablative conditioning. Conditioning regimens for autologous transplantation do not need to be immunosuppressive, since there is greater flexibility in designing protocols, which can provide maximal intensive therapy to eradicate the leukaemia [64].

The most commonly used conditioning regimen for autografting in patients with advanced acute leukaemia is a combination of cyclophosphamide (CY) and total body irradiation (TBI). Another combination, viz., melphalan and single fraction TBI, is used extensively in the treatment of acute leukaemia in the first remission [21,64].

Haematopoietic recovery for patients receiving autologous BMT is slower compared to recovery of patients accepting allogeneic BMT. This is most often because autologous patients who received previous chemotherapy have impaired stem cell quality, which further decreases the supportive effect of the endogenous stroma component [44].

1.12 Peripheral Blood Stem Cell Transplantation

During the past 5 years, cytokine-mobilized (G-CSF) peripheral blood stem cells (PBSC) have replaced bone marrow as the most frequently used source of haematopoietic progenitors for autologous and allogeneic transplantation. For early engraftment after transplantation, cytokines that facilitated haematopoiesis were used. However, some patients show graft failure and die of infectious complications [12,23,46,97].

PBSCs represent a unique population of bone marrow progenitor/stem cells and are released from the bone marrow to reseed damaged marrow spaces. PBSCs circulate in peripheral blood of normal individuals at concentration of 0.1-0.01 of the amount found in the bone marrow. They have been characterized using *in vitro* cultures and the presence of all haematopoietic clonal cells had been demonstrated. The $CD34^+$ cells found in PBSCs are similar to those found in the bone marrow, suggesting that they have the ability to repopulate the haematopoietic system of the irradiated recipient [12,66,97].

PBSCs are more advantageous than bone marrow stem cells because of their lower sensitivity to radiation, increased frequency of cells in the S-phase, delayed logarithmic growth, and increased seeding efficiency [66]. Other advantages of PBSCs over bone marrow cells are their rapidity of engraftment (which diminishes certain transfusion requirements and may also reduce the risk of serious infection) and those stem cells can be collected in the outpatient setting with minimal morbidity [46].

The use of PBSCs for allogeneic transplantation could result in similar benefits. Over the past few years, the clinical use of PBSCs instead of bone marrow was associated with a significant improvement of the haematological recovery after myeloablative chemoradiotherapy in patients with lymphoma, myeloma, and other tumour [28]. Recovery after transplantation includes processes like homing of the infused stem cells into appropriate microenvironment, expansion of their early progenitor cells and differentiation into mature blood cells [52].

1.13 Abnormalities after Transplantation

Myeloablative therapy and transplantation cause abnormalities of the stroma. These abnormalities were demonstrated by an incomplete formation of the stromal layer in the bone marrow obtained from patients undergoing allogeneic BMT and autologous peripheral blood progenitor cell transplantation. These abnormalities were still evident even after six years after transplantation [72,73,80].

There was a selective defect in the ability of bone marrow stroma from patients with AML to support maturation of normal early, uncommitted haematopoietic stem cells ($CD34^+CD38^-$), whilst the ability to support the committed ($CD34^+CD38^+$) cells was not affected [92].

BM stromal cultures derived from patients with CML also displayed functional defects, as indicated by a decreased supporting capacity to normal haematopoiesis. Recently, it had been demonstrated that, like in AML, this dysfunction might be due to the presence of malignant macrophages in the CML-derived stromal cultures [73,80].

Hydroxyurea was demonstrated not to have an effect on stroma function, but when combined with the conditioning treatment before transplantation, it led to defective *in vitro* development of stroma cells from bone marrow. This suggested that it might have been the conditioning therapy that was responsible for the severe deficiencies in stromal growth and supportive ability in cultures from transplanted patients [80].

There was reduced adhesion of stem cells onto leukaemia bone marrow stroma, which led to stem cell loss and decreased marrow recovery [69,70]. Reduction in the clonogenic potential of stroma adherent $CD34^+$ cells suggested that haematopoiesis after transplantation was maintained by reduced numbers of clones [70,72,80].

Certain alterations that were observed in AML patients included a reduced number of fibroblast progenitors (CFU-F) as well as decreased density macrophages and adipocytes [60]. Functional abnormalities were observed in both AML and CML patients because of their inability to possess haematopoietic supportive capacity [72].

AIMS

It has been described that following transplantation, defects in the function of the stroma develop. These defects are associated with decreased adhesion of stem cells to leukaemia bone marrow stroma, which in turn, lead to reduced clonogenic potential of the stroma-adherent $CD34^+$ cells. All these abnormalities alter the process of normal haematopoiesis. Basic fibroblast growth factor (bFGF) is used in this study in attempt to restore this defective haematopoiesis that follow stem cell transplantation.

OBJECTIVES

To evaluate the *in vitro* effects of basic fibroblast growth factor on the:

- Damaged stroma subsequent to transplantation.
- Proliferative capacity of stromal cells after transplantation.
- Stromal supportive capacity for the growth of normal blastic colonies following transplantation.
- Clonogenic potential of stroma-adherent cells after transplantation.

CHAPTER 2

A comparison of marrow stromal proliferative activity in samples drawn from patients who had undergone bone marrow versus peripheral blood stem cells.

Objectives:

- To introduce the importance of bone marrow stroma in haematopoiesis and that any stromal damage may result in inadequate stem cell reserves.
- To determine whether following PBSCs graft infusion, correction of the described abnormalities subsequent to BMT could be detected, given that more mononuclear cells, CFU-GM, and presumably more stem cells were infused after myeloablative conditioning.
- To compare the haematopoietic recovery between patients receiving peripheral blood stem cells (PBSCs) and those patients undergoing bone marrow (BM) transplantation.

2.1 Introduction

Myeloablative therapy followed by allogeneic or autologous bone marrow or stem cell transplantation became standard in a variety of haematological malignancies [70,72]. Usually, recovery of the bone marrow and peripheral blood values ensue within 3-5 weeks of transplantation. However, engraftment in a proportion of patients, may be delayed, whereas in other patients a significant drop in blood parameters may follow infection [6].

Exposure of animals to ionising energy resulted in impairment of the haematopoietic microenvironment and alterations in the bone marrow precursor [29], suggesting persistent reduction in the progenitor cell pool. Using the blastic colony assay, Novitzky et al [70] showed that this was due to significant reductions in the earlier stroma-adherent precursors, while haematopoiesis was maintained through expansion of the committed progeny. It was deduced that despite normalization of blood parameters, there had not been normalization of the stroma-adherent precursors, thus pointing out that the number of progenitors in the graft may need to be maximized.

Allogeneic cytokine-mobilized blood stem cell transplantation has been associated with faster recovery than bone marrow transplantation. This distinction is readily explained by larger proportion of progenitor cell numbers present in the graft of the stem cells.

Table 2.1. Comparison of clinical and demographic data of patients receiving PBSC or BM grafts

	BMT (n = 9)	PBSC (n = 13)	P-value
Median Age (range)	26 (14-39)	41 (23-57)	0.03
Gender Male/Female	4/5	3/10	>0.05
Median MNC x 10^8/kg, (range)	0.9 (0.79-1.35)	7.3 (3.76-13.57)	0.00003
CFU-GM x 10^4/kg	5 (3.5-11)	38.8 (3.1-379)	0.05
Haemoglobin (Hb) g/dl	12.7 (11-13.5)	12.5 (10.9-14.2)	>0.05
Leukocytes x 10^9/l	5.3 (3.6-8.7)	7.1 (3.0-10.2)	>0.05
Polymorphs x 10^9/l	2.36 (1.8-6.6)	4.2 (2.2-8.3)	>0.05
Platelets x 10^9/l	183 (163-335)	211 (166-311)	>0.05

Patients receiving mobilized PBSC grafts were older and studied at a shorter period following transplantation. A significant difference in MNC and CFU-GMs infusion between PBSCT and BMT patients is shown. Significance level was set at <0.05.

2.2 Methods

Twenty-two patients who had received allogeneic stem cell grafts for haematological malignancies were studied. This is a retrospective analysis that included sequential patients who received BMT until 1998 and cytokine-mobilized allogeneic PBSCT thereafter. Thirteen patients received PBSC grafts while nine patients were undergoing BMT. The median age was 41 (range 23-57) for those receiving PBSC and 26 (range 14-39) for BMT patients. The control population was drawn from haematological normal individuals undergoing sternotomy for cardiac surgery under general anaesthesia. The University of Cape Town Ethics Committee approved this study and all patients gave informed consent for bone marrow collections.

Bone marrow aspirates were diluted with α-Minimum Essential Medium (α-MEM) (Gibco, Life Technologies, and UK). Aspirates were layered onto 4 ml Ficoll-Histopaque with a density gradient of 1,077 g/ml (Sigma, St. Louis, and MO) and centrifuged at 400 g for 30 minutes to remove erythrocytes and granulocytes.

Light density population [containing mononuclear cells (MNCs)] was collected from the interface layer, pooled and washed three times with α-MEM to remove Ficoll-Histopaque which is known to be toxic to the cells. MNCs were resuspended in 10 ml of α-MEM and an aliquot was counted on a particle counter (Coulter Electronics, USA). MNCs sample was divided into two aliquots, one to establish bone marrow stromal layers and the other for the selection of $CD34^+$ cells.

2.2.1 Stromal Assay

This assay was performed to measure the proliferative capacity of stromal layer components. A comparison in stromal proliferation between patients receiving PBSC and those undergoing BMT is also evaluated. MNCs were cultured at a concentration of 0.5×10^6/ml in 35 mm petri dishes (Bibby, UK) in α–MEM supplemented with 15% of each foetal calf serum (FCS) and horse serum (Gibco), 2×1^{-6} Mol hydrocortisone (HC), 0.3 μg/ml gentamicin. Stromal cultures were incubated at 37° C in 5% CO_2 and the media changed weekly throughout the culture duration. Stromal confluence was quantitated by placing culture dishes on a grid containing 1 mm squares and scoring the number of squares occupied by stromal layers as a percentage area covered.

2.2.2 Progenitor Cell Selection

This procedure was implemented to select haematopoietic progenitors that express the $CD34^+$ antigen on their cell surface membranes. The second aliquot of bone marrow MNCs was incubated in 150 mm petri dish (Bibby, UK) for 2 hours at 37°C in 5% CO_2 to remove monocytes. Non-adhesive population of cells from dishes were lymphocyte-depleted by addition of Campath 1M (6.25 mg/ml; 0.01 ml antibody/5×10^6 cells/ml) in the presence of 30% fresh AB serum as a source of complement and then incubated in 150 mm petri dish for 50 minutes at the same conditions as above.

The lymphocyte and monocyte poor cell population was suspended in cold (4°C) PBS and exposed for 30 minutes to paramagnetic beads covalently bound to anti-CD34 monoclonal antibody (Dynal, Olso) on an apparatus that provides both the tilting and rotation. Rosetted cells were recovered with a magnetic particle concentrator (MPC), and then released from beads by incubation with a "Detachabead" (polyclonal murine immunoglobulin: Dynal, Oslo). Selected $CD34^+$ cells were resuspended in 1 ml of 2% AB serum/PBS solution and a $CD34^+$ count performed manually using a Neubauer counting chamber and adjusted to a concentration of 1×10^4/ml.

2.2.3 Colony Forming Unit–blast (CFU-bl) Assay

This assay was performed in order to compare the stromal supportive capacity and the clonogenic potential of selected $CD34^+$ cells between patients receiving BM or PBSC grafts. Selected 1×10^4 $CD34^+$ cells were suspended in 1 ml of α-MEM supplemented with 15% FCS, layered in duplicate onto preformed stroma in cross-culture studies and incubated for 2 hours to allow optimal adherence. The stroma non-adherent cells were removed by standard washing with Iscove's Modified Dulbecco's Medium (IMDM) and cultured in colony forming unit-granulocyte macrophage (CFU-GM) assay.

Stroma-adhered cells were covered with 0.3% agar, α-MEM supplemented with 30% FCS, and were incubated at 37°C in 5% CO_2 for 5 days. Aggregates containing more than 20 cells were counted as blastic colonies on day 6 using an inverted microscope. When stroma was not confluent, CFU-bl results were expressed by multiplying the colony number obtained by 100 and divided by the percentage surface area covered (CFU-bl x 100/% surface covered).

2.2.4 Colony Forming Unit-Granulocyte Macrophage Assay

This assay was carried out to measure the clonogenic and proliferative capacity of selected $CD34^+$ cells between patients receiving either BM or PBSC grafts. Stroma non-adherent $CD34^+$ cells were cultured in the presence of 10 ng/ml GM-CSF, 30% FCS, and 0.3% agar. They were incubated for 14 days at 37°C in 5% CO_2. For permanent recording, CFU-GM cultures were fixed with 0.25% glutarylaldehyde for 7 minutes and then floated in weighing boats containing water. The pellicles were mounted on the slide and stained with May-Grünwald-Giemsa (MGG). Using an inverted microscope, aggregates of more than 40 cells were counted as CFU-GMs.

2.3 Results

Patients' demographic data and blood parameters at study are given in Table 2.1. The median time from transplantation to the investigation of the marrow populations was 12 months (range 6-12). In the PBSC group, grafts contained a median of 1.2 (range 0.7-2.2) $CD34^+$ cells/kg, while similar data for patients undergoing BMT was not available.

Table 2.2. Comparison in stromal confluence, blastic colony, and non-adherent cell results between controls, PBSCT, and BMT patients

	Stroma: % area covered	CFU-bl	CFU-GM	CFU-bl/CFU-GM
Control				
median	98	98	99	
(range)	(76-100)	(38-200)	(19-221)	0.9
	##p= 0.0001	##p= 0.003	p= 0.4	##p= 0.04
PBSC				
median	65	47	96	0.3
(range)	(40-80)	(8-121)	(45-215)	
	**p= 0.4	**p= 0.2	p= 0.3	**p= 0.06
BMT				
Median	30	12	46	0.4
(range)	(0-80)	(2-133)	(5-186)	

*Data represents results of the percentage area covered by stroma, blastic colony and CFU-GM between PBSCT and BMT patients. The last column gives the ratio between CFU-bl and CFU-GM. ## denotes significance difference between patient and control population, while ** indicates significant values between both stem cell sources.*

2.3.1 Appearance of Bone Marrow Stroma

Quantitation of stromal monolayers confluence was determined at 4-6 weeks from initiation of cultures. The median area covered by stroma in normal individuals was 98% (range 76-100) while the median area of the dish covered from both stem cell sources was 47.5% (range 0-80%). Both stem cell sources showed significantly less growth compared to the median area covered by cells from normal individuals (p= 0.0001; Table 2.2).

2.3.2 CFU-bl and CFU-GM

Within the transplant group, $CD34^+$ cells from patients who had received mobilized PBSC grafts cultured on normal stroma had higher CFU-bl scores than those originating from BMT recipients, but the differences were not significant ($p = 0.2$). Both PBSC and BMT $CD34^+$ cells generated fewer CFU-bl than selected normal cells (12 and 47 vs. 98%; $p=0.003$; Table 2.2).

In cross-culture experiments, stroma from patients who had recovered from both sources of stem cell transplantation supported less well normal stroma-adherent $CD34^+$ cells than did control stromal layers (21.5 vs. 97.5 CFU-bl; $p = 0.0003$). However, when control 1×10^4 $CD34^+$ cells were layered onto stroma from patients receiving PBSC or BM, significantly more colonies were scored on stromal layers originating from patients who received PBSCT (median 39, range 2-121) than those who received BMT (median 5, range 0-59; $p = 0.03$). The median colony numbers from both sources of patients was 29.5 (range 2-133) and these scores were significantly lower than blastic colonies from normal individuals (median 98; $p=0.03$; Table 2.2).

Stroma non-adherent $CD34^+$ cells eluted after a 2-hour incubation from patients' stromal layers and cultured in CFU-GM assay led to median values of colonies that were not significantly different from the control group. However, the ratio of CFU-bl/CFU-GM within the $CD34^+$ population confirmed a significant reduction in the proliferation of CFU-bl that was compensated by a larger proportion of CFU-GM and normalization of the blood values (Table 2.1).

In summary, despite similar conditioning and adequate recovery of blood parameters, individuals receiving PBSC grafts formed significantly more extensive stroma and when corrected for surface area, they supported greater numbers of normal CFU-bl than those receiving BM grafts. The normalization of blood parameters may have occurred from significant amplification of the committed (stroma non-adherent) progenitors monitored using the CFU-GM assay.

2.4 Discussion

Using stroma dependent assays, Novitzky et al [70], observed that following autologous or allogeneic BMT, a derangement in the proliferation and function of the bone marrow stroma and of the selected $CD34^+$ bone marrow progenitors was noted. They documented a reduction in the clonogenic potential of the $CD34^+$ population, which does not recover even 8 years after BMT [70].

In the current study, we compare the results of patients who had received two different sources of stem cell transplantation. The results show that cells derived from patients receiving cytokine-mobilized PBSC transplants express a modest recovery in CFU-bl and a significant expansion in the area of the dish covered by marrow stromal layers. Normalization of the blood values appear to be associated with active proliferation of the more committed population of stroma-adherent cells, as performed in CFU-GM assay (Table 2.1).

Recovery in the stroma is associated with greater proliferation of normal $CD34^+$ stroma-adherent precursors leading to higher CFU-bl numbers. These improvements are only noted when normal sources of stem cells are used, not with stem cells from patients who had received transplantation.

Faster recovery of blood parameters with PBSC grafts may be related to the presence of larger numbers of committed precursors that rapidly differentiate into functional elements, while the most primitive self-replicating ancestors that develop slowly may be released in more limited proportions. As these studies were performed at a median of 6 months following infusion of the graft, recovery of the progenitor cell population might have not been complete. This is unlikely as no difference between those tested at 6 or 12 months post-transplantation was noted [72].

To enhance the haematopoietic recovery after stem cell transplantation, a number of strategies to improve mobilization of $CD34^+$ cells to the blood have been attempted. It is of particular note that large numbers of endothelial [86], lymphoid, monocytoid and other undefined populations together with haematopoietic precursors may also be harvested. The cytokine-mobilized PBSC harvest may also be enriched for elements that are critical for the recovery of the bone marrow microenvironment. Investigations to demonstrate these populations were not performed in this study.

In a similar way, PBSCs have higher numbers of CFU-GM. Furthermore, the addition of stromal cells in the foetal sheep model showed that donors' stromal elements repopulated marrow spaces and this facilitated donor stem cell engraftment. A direct benefit in survival of patients receiving PBSC grafts, where cytokine-mobilized blood cells that allowed a faster engraftment were used, has not been documented [86].

Even though blood counts in both groups normalized, two issues are still of practical relevance. *Firstly*, despite increases in the number of $CD34^+$ cells in the graft above a threshold of 5×10^6 cells/kg, the rate of engraftment has not been shortened beyond 9 days from transplantation and this delay may be directly dependent on the integrity of the stroma.

Secondly, there is evidence that the transplantation procedure may compromise the longevity of the haematopoietic stem cells, as assessed by re-transplantation assays in animals or shortened telomeric sequences in leukocytes from patients receiving allogeneic transplants as compared to their donors [51].

2.5 Conclusion

In the current study we show that the bone marrow stroma from patients receiving mobilized progenitor cells proliferated better than patients receiving bone marrow grafts. We postulate that elements that are important for the integrity of the bone marrow microenvironment may also be released into the circulation.

It would be of importance to establish if a healthier microenvironment may lead to improvement in the recovery of the transplanted stem cell population. This may be of particular significance to patients who are receiving repetitive courses of chemotherapy that are damaging their haematopoietic and mesenchymal cell progenitors.

CHAPTER 3

The effects of basic Growth Factor on the clonogenic potential of normal $CD34^+$ cells.

Objectives:

- To establish if bFGF has a direct clonogenic effect on selected CD 34^+ cells.
- To establish the effects of low and high concentrations of bFGF with other growth factors on $CD34^+$ cells.

3.1 Introduction

Cytokines are a group of proteins that have potent biological activities. Activation of cells by cytokines is mediated by an interaction with specific receptors expressed on the cell membrane surfaces, thereby triggering intracellular events leading to a specific action. Most cytokines have pleiotropic effects, meaning that in addition to the stimulation of cellular proliferation, they also mediate a wide range of other activities e.g., wound healing. Cytokines induce cell proliferation by affecting the expression of genes involved in normal growth control pathways.

Pleiotropic cytokines such as interleukin (IL)-3 and stem cell factor (SCF) stimulate cells of the myeloid lineage. Activity of cells is mediated by interaction of cytokines with specific receptors expressed on the surfaces of target cells, thereby triggering intracellular events leading to an observed cytokine action [99]. These cytokines can be grouped into early-acting factors like IL-3 and SCF, and late-acting lineage-specific factors such as colony stimulating factor-granulocyte macrophage (GM-CSF) and erythropoietin (Epo). IL-3 is a multipotent cytokine that stimulates primitive and also lineage-committed progenitors. SCF acts in combination with cytokines like IL-1and IL-6 to support multilineage colony formation. GM-CSF promotes the formation of myeloid colonies, while Epo is responsible for differentiation and maturation of erythroid cells [74].

Basic Fibroblast Growth Factor (bFGF) is a cytokine with pleiotropic effects on multiple cell types constituting the haematopoietic tissue. Basic FGF induces proliferation of bone marrow stromal cells and enhances the myeloid cell numbers as shown by the elevation in the number of adherent and non-adherent layer derived progenitor cells. It was noted that bFGF augments the effects of other cytokines on granulocyte-monocyte, erythroid, or mixed lineage colony formation. Basic FGF and SCF are synergistic in the regulation of the proliferation of adjacent haematopoietic stem cells [10,34].

3.2 Methods

Seven controls from the 31 normal individuals (Appendix B) who are undergoing sternotomy for cardiac surgery were studied (controls 15-22, except for control 21). Their median age was 42.8 (range 19-64). Mononuclear cells (MNCs) were collected from the interface layer, washed three times with α-MEM, monocyte-depleted by means of plastic adherence and lymphocyte-depleted by addition of Campath 1M and complement. Refer to chapter 2 for a detailed method.

3.2.1 Sources of Growth Factors

Basic fibroblast growth factor was bought from Scio's Inc., Mountain View; CA. Epo was donated by Bioclones (Sandton, Gauteng, South Africa) and once reconstituted to 1000 U/ml kept at 4°C. Recombinant human cytokines were bought from Amgen, Thousand Oaks, CA; rhesus-IL-3 had a specific activity of 1×10^7 U/mg, rhesus-GM-CSF, G-CSF, or SCF had a specific activity of 1×10^8 U/ml each, at a protein concentration of 0.5, 0.5, and 2 mg/ml respectively, and were diluted to 1 μg/ml and stored at 4°C until used.

3.3.2 Progenitor Cell Selection

Refer to chapter 2 for a detailed method for the selection of $CD34^+$ cells.

Table 3.1. Different cytokine combinations used in clonogenic assay

Culture Number	Cytokine Combinations
1	bFGF 2
2	bFGF 20
3	Epo 1
4	Epo 1, bFGF2
5	Epo 1, bFGF 20
6	Epo 1, $IL_3$10
7	Epo 1, $IL_3$10, bFGF 2
8	Epo 1, $IL_3$10, bFGF 20
9	Epo 1, GM10, bFGF 2
10	Epo 1, GM10, bFGF 20
11	Epo 1, $IL_3$10, SCF 20
12	Epo 1, $IL_3$10, SCF 20, bFGF 2
13	Epo 1, $IL_3$10, SCF 20, bFGF 20

Epo= erythropoietin (1ng/ml), bFGF= basic fibroblast growth factor, $IL_3$10= Interleukin-3 (10 ng/ml), GM 10 = colony stimulating factor-granulocyte-monocyte (10ng/ml), SCF 20= stem cell factor (20 ng/ml).

3.3.2 Clonogenic Assay

Selected $CD34^+$ cells were cultured at concentrations between 1-5 x 10^3 in the presence of $5x10^{-5}$ M 2 mercaptoethanol (ME), gentamicin and 30% FCS in the presence of the above-mentioned growth factors (Table 3.1). An aliquot of 0.25 ml of this suspension was added to each of the four-well dishes (Nunclon, Nunc, Denmark), covered with 0.3% agar and incubated for 14 days at 37°C in 5% CO_2.

For permanent recording, cultures were fixed in 0.25% glyteraldehyde for 7 minutes, mounted onto microscopic slides, and stained with MGG. Using an inverted microscope, burst forming unit-erythroid (BFU-E) were recognized as haemoglobinised colonies composing of more than 99 cells or when aggregates consist of three or more subclusters. CFU-E were described as erythroid clusters of 4-99 cells and CFU-GMs were defined as myeloid aggregates containing more than 40 cells.

3.4 Statistical Analysis

The program Basic Statistica was used to analyze the results and a specific analysis tool used was the t-test for dependent variables. Significance level was set at <0.05. Bar charts were constructed based on averages obtained from all the results.

3.5 Results

Of the total selected cell population a mean of 92.4% cells expressing $CD34^+$ antigen were documented. These cells were cultured in duplicate at 5 x 10^3 /ml, in the presence of defined combinations of cytokines (Table 3.1).

Figure 3.1. Effect of bFGF on CD34$^+$ cells

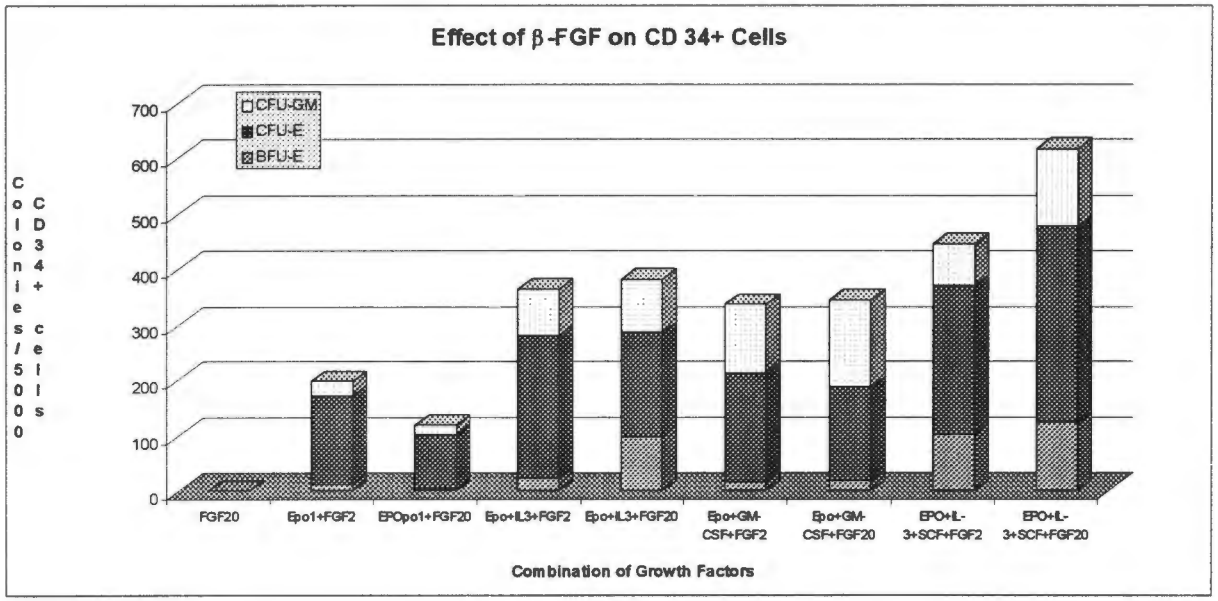


Figure 3.1. *Response of selected CD34$^+$ to bFGF alone and in combination with SCF, GM-CSF, IL-3 and Epo. A concentration of 5 x 10^5/ml cells were cultured for 14 days, mounted on slides and stained with MGG. Data represents bars constructed from medians. The top parts of the bars represent the CFU-GMs, the middle parts represent CFU-Es and the bottom parts represent the BFU-Es.*

Table 3.2. Results of the effects of bFGF on the clonogenic growth of $CD34^+$ cells

Cytokine Concentrations	Cultures	Colony Numbers (Averages)	Statistical Analysis (P=)
bFGF 2	ALL	No colonies	
bFGF 20	ALL	No colonies	
Epo 1	BFU-E	19.5	
	CFU-E	108	
Epo 1, bFGF 2	CFU-GM	27.5	
	BFU-E	23	0.2
	CFU-E	212.5	0.4
Epo 1, bFGF 20	CFU-GM	39.5	
	BFU-E	26.25	0.3
	CFU-E	163.25	0.7
Epo 1, IL_3 10	CFU-GM	44	
	BFU-E	204	
	CFU-E	202	
Epo 1, IL_3 10, bFGF 2	CFU-GM	110.5	0.6
	BFU-E	77.25	0.5
	CFU-E	270.25	0.5
Epo 1, IL_3 10, bFGF 20	CFU-GM	109.75	0.5
	BFU-E	167.5	0.6
	CFU-E	245.75	0.3
Epo 1, GM 10, bFGF 2	CFU-GM	209	
	BFU-E	29.25	
	CFU-E	252.25	
Epo 1, GM 10, bFGF 20	CFU-GM	272.75	0.1
	BFU-E	26.5	0.8
	CFU-E	178.25	0.3
Epo 1, IL_3 10, SCF 20	CFU-GM	34	
	BFU-E	228	
	CFU-E	60	
Epo 1, IL_3 10, SCF 20, bFGF 2	CFU-GM	142.5	0.4
	BFU- E	122	0.8
	CFU-E	264.5	0.1
Epo 1, IL_3 10, SCF 20, bFGF 20	CFU-GM	166	0.2
	BFU-E	146.25	0.7
	CFU-E	342.5	0.2

Data represents the clonogenic growth from 5 x 10^5 cultured cells scored after 14 days. ALL denotes that there was no growth in any of the cultures. Different colony numbers (CFU-GM, BFU-E and CFU-E) are compared with each other when the 0, 2 and 20 ng bFGF concentrations are used. Statistical analysis was performed by the t-test for dependent variables. Significance level was set at <0.05. Epo= erythropoietin (1ng/ml), bFGF= basic fibroblast growth factor, $IL_3$10= Interleukin-3 (10 ng/ml), GM 10 = colony stimulating factor-granulocyte-monocyte (10ng/ml), SCF 20= stem cell factor (20 ng/ml).

3.5.1 Results of BFU-Es.

When 5 x 10^3 $CD34^+$ cells were exposed to bFGF alone, no clonogenic growth was noted (Table 3.2 and Figure 3.1). When similar numbers of cells were exposed to Epo and bFGF (2 and 20 ng/ml), erythroid bursts were increased, even though not significantly increased above those obtained in the presence of Epo alone (p= 0.2 and 0.3). Results show that the low and high concentrations of bFGF did not augment the proliferation of immature erythrocytes, even when added with other growth factors (Table 3.2 and Figure 1).

3.5.2 Results of CFU-Es

Addition of Epo and bFGF (2 and 20 ng/ml) to cultures resulted in an insignificant augmentation in erythroid colony growth above those in the presence of Epo alone (p= 0.4 and 0.7). Results in Table 3.2 demonstrate that bFGF was unable to increase the colony numbers of mature erythrocytes even when supplemented with other growth factors.

3.5.3 Results of CFU-GMs

Myeloid colonies were insignificantly lower in cultures supplemented with Epo, IL-3 and SCF when compared to those found in the presence of Epo, IL-3, SCF and bFGF. Myeloid colonies in the presence of Epo, CSF-GM and bFGF 20 ng/ml were also insignificantly increased above colonies obtained in cultures treated with Epo, CSF-GM and bFGF 2 ng/ml (p=0.1; Table 3.2). Data demonstrate that the low and high concentrations of bFGF did not expand the number of myeloid colonies, even when added in conjunction with other growth factors.

3.6 Discussion

No colony growth is observed when bone marrow derived $CD34^+$ cells are grown in the presence of bFGF These results correspond to those obtained by Berardi A. C, 1995 [10]. Our results also show that there is no difference in all colony numbers when bFGF is added at low or high concentrations. There was no significant expansion of $CD34^+$ cells when FGF was added to other growth factors.

It is known that proliferation of early and committed progenitors needs stimulation by combination of growth factors, but results obtained from this chapter demonstrate that bFGF in combinations with 3 or 4 other growth factors was unable to elicit a significant augmentation on colony numbers. Although, SCF and bFGF have shown to be synergistic in expanding committed myeloid progenitor cell growth [34], this study was unable to confirm these observations.

3.7 Conclusion

Results demonstrate that:

- bFGF is unable to elicit an effect on the clonogenic growth of $CD34^+$ cells, when it is added on its own to cultures.
- bFGF at any concentration, is unable to induce significant proliferation of $CD34^+$, even when added in combination with other growth factors.

CHAPTER 4

The effects of bFGF on normal fibroblastic progenitors and on the ability of normal stromal layers to support the clonogenic ability of $CD34^+$ and the development of blastic colonies

Objective: To obtain the optimum concentration to be used on patients who have undergone bone marrow transplantation, by using varying concentrations of bFGF on a normal population.

4.1 Introduction

In each assay the 0.2, 2 and 20 ng/ml bFGF with and without heparan sulphate were added in duplicate (Table 4.1). Heparan sulphate was added or omitted from cultures in order to determine if it has any effect on normal cells when added in conjunction with bFGF. Cultures not supplemented with either bFGF or heparan sulphate were regarded as control samples. Two different assays were employed: the CFU-Fs and stroma, as measured by the surface area covered by stroma and cell numbers enumerated after trypsinization of monolayers.

Table 4.1. Different bFGF concentrations used in cultures

Culture Number	bFGF Concentration
1	Control
2	0.2 ng bFGF + 20 ng HS
3	0.2 ng bFGF
4	2 ng bFGF and 20 ng HS
5	2 ng bFGF
6	20 ng bFGF + 20 ng HS
7	20 ng bFGF

bFGF concentrations were added in the absence and presence of HS. ng= nanograms and HS stands for heparan sulphate.

4.2 Methods

Bone marrow aspirates were drawn from thirty-one haematologically normal individuals undergoing sternotomy for cardiac surgery under general anaesthesia. The median age for this control group was 48 years (range 19 – 74; Appendix B). Informed consent for bone marrow collections was obtained.

4.2.1 Stromal Assay

This assay was carried out to evaluate the effects of bFGF on the proliferation of normal stromal cells. MNCs were cultured in the absence and presence of bFGF supplemented with heparan sulphate (Table 4.1). Refer to chapter 2 for a description of the method. Investigations of the effect of bFGF in a dose-response study on the stromal confluence was done by placing culture dishes on a grid containing 1-mm squares and scoring the number of squares occupied by stromal layers as percentage area covered.

4.2.2 Fibroblastic Progenitor (CFU-Fs) Assay

This assay was performed to assess the effects of bFGF on normal fibroblastic progenitors. Bone marrow MNCs were cultured at a concentration of 1 x 10^6/5ml in the absence and presence of bFGF in 50 mm petri dishes. Fibroblastic progenitors were isolated from bone marrow cells by culturing MNCs in α-MEM supplemented with 20% FCS. Fibroblastic cells were incubated at 37°C in 5% CO_2 and media were changed on day 5. After 9 days of culture, supernatants were removed from the dishes, air-dried, fixed and stained with MGG. Aggregates of more than 20 cells were defined as CFU-Fs using an inverted microscope.

4.2.3 Progenitor Cell Selection

Refer to chapter 2 for the description of the method for the selection of $CD34^+$ cells. Aliqouts of the selected cells were studied by the standard flow cytometry.

4.2.4 CFU-bl Assay

This assay was performed to evaluate the effects of bFGF on the normal stromal capacity to support the clonogenic potential of $CD34^+$ cells and the growth of normal blastic colonies. Selected $CD34^+$ cells were layered on preformed stromal layers that have been treated with or without bFGF. Refer to chapter 2 for the description of the method.

4.3 Statistical Analysis

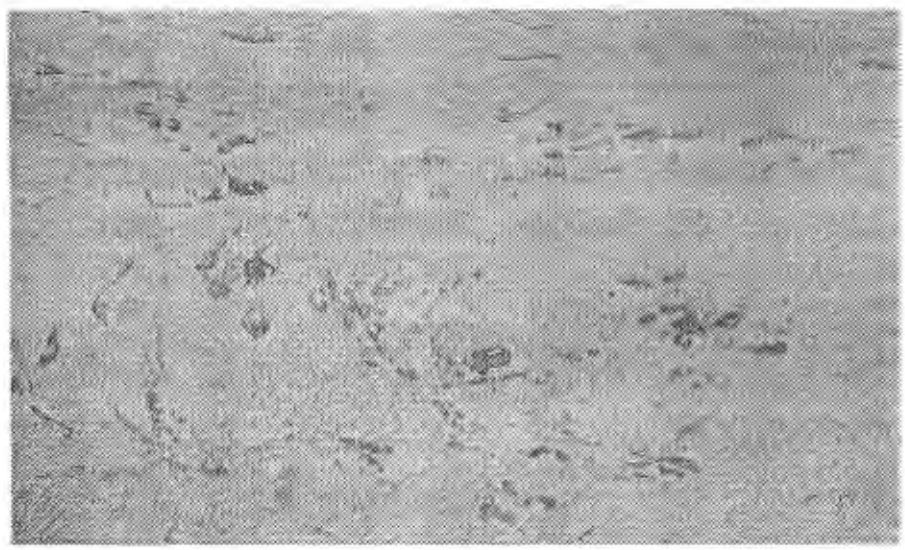
Statistical analysis was performed by the paired Student t-test. The Student t-test was used to demonstrate the study groups' distinction before and after the treatment. Significance level was set at <0.05.

4.4 Results

4.4.1 Appearance of Bone Marrow Stroma

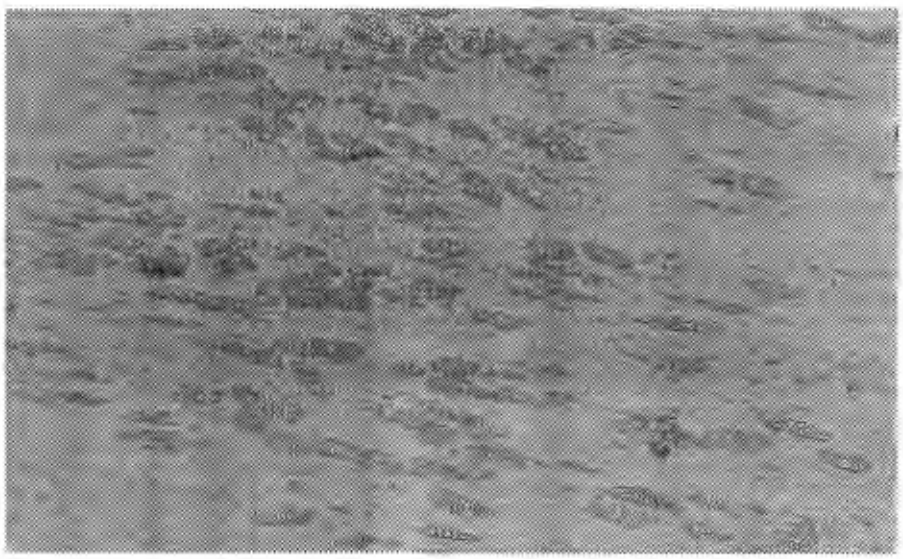
Stromal confluence was scored at 3 weeks of culture, since stromal layers cultured in the presence of bFGF appeared to be confluent within 3 weeks.

Picture 4A



Picture 4A. *A stromal layer from a normal individual, cultured from 0.5 x 10^6/ml MNCs for 3 weeks in the absence of bFGF.*

Picture 4B



Picture 4B. *Stromal layer from a normal individual, cultured* ***from 0.5 x 10^6/ml MNCs*** *for 3 weeks in the presence of bFGF.*

Stromal layers cultured in the absence of bFGF grew into a monolayer (Picture 4A), whilst stromal layers cultured in the presence of bFGF grew at high densities, in multilayers and different directions (Picture 4B), as shown by Wilson, et al, 1991 [103]. Microscopically, fibroblasts in stroma supplemented with bFGF were more elongated and formed a denser background than fibroblasts in stromal layers cultured in the absence of bFGF (Picture 4B vs. 4A). Fat cells in bFGF-treated cultures started becoming visible after 2 weeks of culture and appeared increased in number and size (Picture 4B).

Table 4.2. The effect of bFGF on the proliferation of normal bone marrow stromal cells

Culture	% Confluence (Median)	Range	P=Value
Control	55	30-65	
0.2 ng bFGF + HS	47.5	35-60	NS
0.2 ng bFGF	50	30-60	NS
2 ng bFGF + HS	70	50-95	<0.05
2 ng bFGF	65	50-80	0.0006
20 ng bFGF + HS	80	65-99	0.004
20 ng bFGF	77.5	70-98	<0.05

Mononuclear cells 0.5 x 10^6/ml were incubated in the absence and presence of varying bFGF concentrations, in α-MEM supplemented with 15% of each FCS and horse serum, 2 x 10^{-7} HC for 21 days. Data represents median confluence in percentages, ranges and p-values. NS represents values that are not significant.

Mononuclear cells from normal individuals were cultured at a concentration of 0.5 x 10^6/ml in 35 mm dishes for 3 weeks, in the absence and presence of the above-mentioned bFGF concentrations (Table 4.1). The median surface area covered by stromal layers was 55% (range 30-65), and the area covered was significantly improved with the addition of 2 and 20 ng/ml bFGF with and without 20 ng/ml heparan sulphate (Table 4.2).

4.4.2 Stromal Cell Numbers

Table 4.3. The effect of bFGF on the proliferation of bone marrow stromal cells

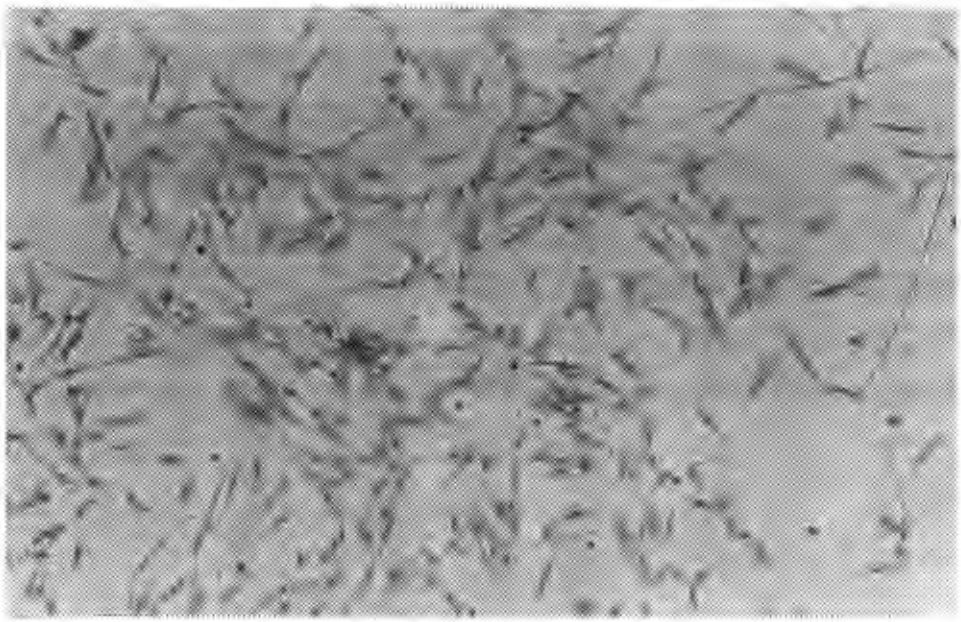
Culture	Cell Number x 10^6/2ml	Range	P=Value
Control	0.61	0.15-1.66	
0.2 ng bFGF + HS	0.61	0.12-1.67	NS
0.2 ng bFGF	0.83	0.32-1.99	NS
2 ng bFGF + HS	1.27	0.27-4.11	0.03
2 ng bFGF	0.83	0.29-1.36	NS
20 ng bFGF + HS	1.47	0.44-1.89	0.03
20 ng bFGF	1.09	0.33-1.76	NS

Cells 0.5 x 10^6/ml cultured in duplicate, supplemented with various bFGF concentrations formed stromal layers for 21 days and were incubated with 5% trypsin solution for 10 minutes. Stromal cell numbers are expressed as medians and significant values are given by the paired t-test. NS stands for not significant.

Single cell suspension from trypsinized stromal layers was prepared to determine the effects of various concentrations of bFGF. Stromal cell numbers in the dishes were enumerated with a particle counter. Stromal layers established at 0.5 x 10^6/ml mononuclear cells produced 0.61 x 10^6/2ml cells (range 0.15-1.66). Stromal cells increased significantly upon the addition of 2 and 20 ng/ml with 20 ng/ml heparan sulphate (p=0.03 each; Table 4.3)

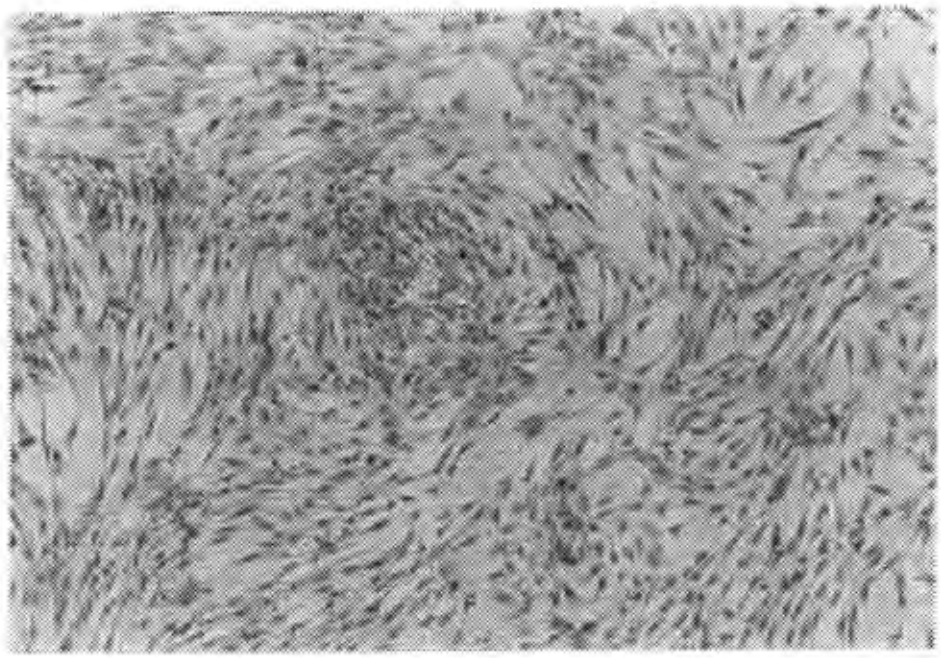
4.4.3. Fibroblastic Progenitors (CFU-Fs)

Picture 4C



Picture 4C. *Fibroblastic progenitor from a normal individual cultured at 1 x 10^6/5ml for 9 days in the absence of bFGF and stained with MGG.*

Picture 4D



Picture 4D. *Fibroblastic progenitors from a normal individual cultured at 1 x 10^6/5ml for 9 days in the presence of bFGF and stained with MGG.*

Basic FGF increased fibroblastic colony sizes, but the colonies expanded and became confluent in such a way that it was very difficult to quantitate them (Picture 4D), unlike the discrete fibroblastic colonies obtained in the absence of bFGF (Picture 4C).

Table 4.4. The effect of bFGF on the growth of fibroblastic progenitors

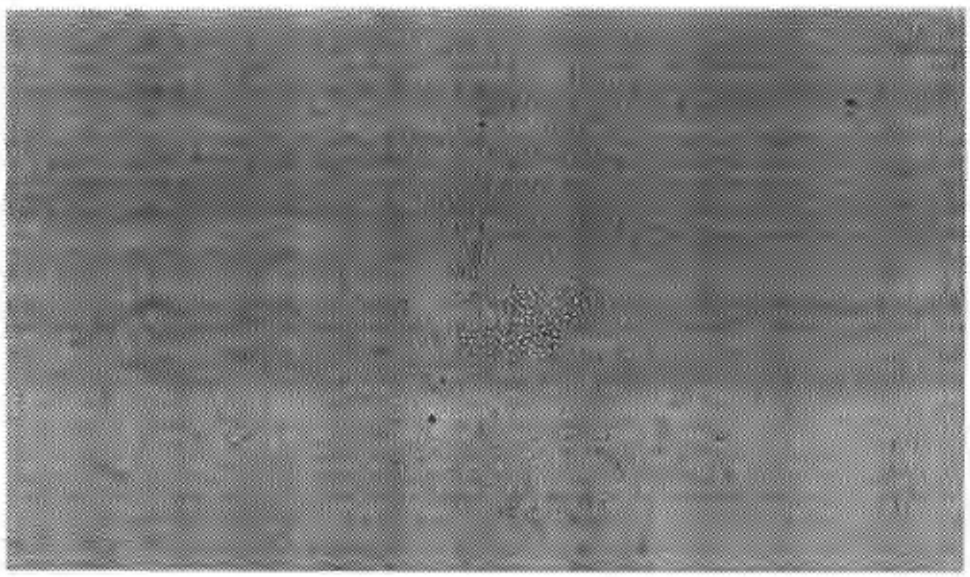
Culture	CFU-F x 10^6/5ml	Range	P-Value
Control	28.3	14-53	
0.2 ng bFGF + HS	28.0	3-67	NS
0.2 ng bFGF	18.5	12-25	NS
2 ng bFGF + HS	23.6	14-36	NS
2 ng bFGF	19.9	11-34	0.05
20 ng bFGF + HS	23.0	7-40	NS
20 ng bFGF	16.2	5-28	0.05

Fibroblastic progenitors were established at a concentration of 1 x 10^6/5ml in α-MEM supplemented with 20% FCS for 9 days. Fibroblastic colony results are expressed as medians and the significance level was set at <0.05.

Following 9 days of incubation, fibroblastic cultures were terminated, fixed and stained with MGG and colony numbers scored using an inverted microscope. Fibroblastic colony numbers were significantly increased in control samples (median 28.3 x 10^6/5ml CFU-Fs; range 14-53), but the addition of bFGF to cultures did not change the number of fibroblastic colonies. The highest concentration of bFGF appeared to be inhibitory on fibroblastic colony numbers (Table 4.4).

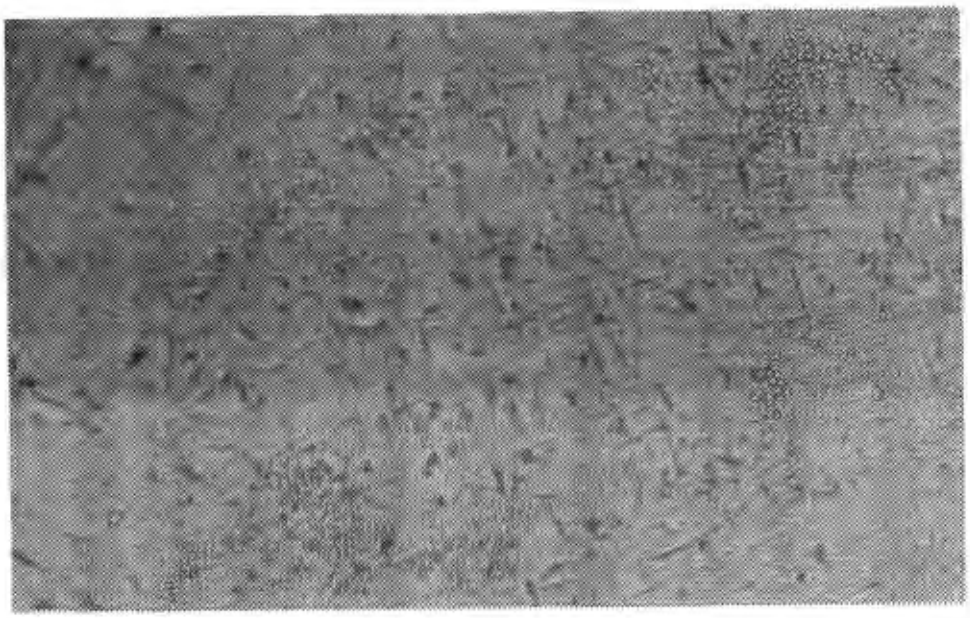
4.4.4. Blastic colonies (CFU-bl)

Picture 4E



Picture 4E. *A blastic colony (centre) obtained when normal $CD34^+$ cells were panned on normal stroma in the absence of bFGF.*

Picture 4F



Picture 4F. *Blastic colonies obtained when normal $CD34^+$ cells were cultured on normal stroma that was supplemented with bFGF.*

Stroma-adherent cells forming a blastic colony on a stromal layer that was cultured in the absence of bFGF were small and loose (Picture 4E). Normal blastic colonies obtained from bFGF-treated stromal layer were increased in number and larger than those found in control cultures (Pictures 4E vs. 4F).

Table 4.5. The effect of bFGF on normal stroma-adherent colonies

Culture	CFU-bl x 10^4/ml	Range	P-Value
Control	121.8	3-184	
0.2 ng bFGF + HS	104.0	21-159	NS
0.2 ng bFGF	106.1	16-218	NS
2 ng bFGF + HS	132.8	41-260	NS
2 ng bFGF	107.3	7-176	NS
20 ng bFGF + HS	156.8	43-271	0.03
20 ng bFGF	114.5	8-207	0.01

Monocyte and lymphocyte depleted selected 1 x 10^4 $CD34^+$ cells were layered in duplicates, in cross-culture assays on normal preformed stromal layers. Cells were incubated for 2 hours to allow optimal adherence. Stroma non-adherent cells were washed off the dishes and stroma-adherent cells were covered with 0.3% agar and cultured for 6 days. Blastic colony results are expressed as medians.

Selected normal cells expressing $CD34^+$ antigen had a mean percentage of 92.4% of the total selected cell population. The median number of blastic colonies from normal individuals in the absence of bFGF was 121.8 x 10^4/ml CFU-bl (range 3-184). This score was significantly higher upon the addition of 20 ng/ml bFGF with 20 ng/ml heparan sulphate (median 156.8 vs. 121.8 CFU-bl; range 43-271), but significantly lower with the addition of 20 ng/ml bFGF without 20 ng/ml heparan sulphate (median 114.5 vs. 121.8 CFU-bl; Table 4.5). Basic FGF concentration without heparan sulphate was inhibitory.

4.5 Discussion

Results show that there is a greater increase in the proliferation rate of normal stromal layers in the presence of bFGF than the rate of normal stromal layers cultured in the absence of bFGF. There is no significant effect on the surface area covered when the 0.2 ng/ml bFGF with and without 20 ng/ml heparan sulphate is added to cultures. Heparan sulphate has an incremental effect on the surface area covered by stroma when it is added in conjunction with bFGF, since stromal layers cultured in the presence of bFGF and heparan sulphate grow better than those stromal layers cultured in the presence of bFGF alone (without heparan sulphate).

Stromal cell numbers from cultures treated with bFGF were significantly increased above those cell numbers from control cultures with no bFGF. Basic FGF concentrations supplemented with heparan sulphate have a more significant effect on stromal cell numbers than those bFGF concentrations without heparan sulphate, indicating that heparan sulphate has a major additive effect on stromal cell numbers when added in conjunction with bFGF.

Results show that even though bFGF is unable to increase fibroblastic colony numbers above those of the controls, it has an effect on the sizes of the colonies, as they become larger in the presence of bFGF than colonies in the absence of bFGF.

Addition of 20 ng/ml bFGF accounts for greater proliferation of normal stroma-adherent $CD34^+$ cells, leading to higher blastic colony scores above those colony numbers in bFGF untreated cultures.

4.6 Conclusion

Data from this chapter demonstrates that:

- bFGF does not increase the number of fibroblastic progenitors, but only augments colony sizes.
- 0.2 ng/ml bFGF is ineffective in all the culture assays even when added in conjunction with heparan sulphate.
- 2 and 20 ng/ml with 20 ng/ml heparan sulphate elevate both proliferation rate of stromal layers to be confluent within 3 weeks and also increase stromal cell numbers.
- 20 ng/ml bFGF with 20 ng/ml heparan sulphate significantly augments blastic colony numbers and increases their colony sizes.

The effects of 2 and 20 ng/ml bFGF with 20 ng/ml heparan sulphate will then be studied in vitro on patients who have undergone either autologous or allogeneic peripheral blood stem cell transplantation, in order to evaluate whether they will be able to repair impaired haematopoiesis.

CHAPTER 5

Effects of bFGF on the bone marrow microenvironment in the recovery stage following transplantation.

Objectives:

- To evaluate the modulatory effects of bFGF when it is added to growing stromal layers and fibroblastic progenitors.
- To assess the effects of bFGF on the defective stromal supportive capacity and reduced clonogenic potential of bone marrow derived $CD34^+$ cells following PBSCT.
- To compare the results obtained from this chapter to those found in normal individuals.

5.1 Patient Population

Sixteen subjects with blood disorders who had undergone autologous (n=3) or allogeneic (n=13) peripheral blood stem cell transplantation (PBSCT) were studied. Patients were studied at 6 and 12 months in complete remission of their blood disorders following PBSCT and a control group was drawn from haematologically normal individuals undergoing sternotomy for cardiac surgery. None of the patients or normal individuals had a history of recent inflammation or infectious disorder at the time of investigation. Informed consent was always acquired from patients according to the guidelines provided by the Ethics and Research Committee of the University of Cape Town and Groote Schuur Hospital. The median age of patients was 41.5 years (range 17-57) while the median age of normal individuals was 48 years (range 19-74). Refer to Table 5.1 for clinical data of patients.

Table 5.1. Clinical data of patients

Patient Diagnosis	Number of Patients	Type of Transplantation
Acute Myeloid Leukaemia	2	Autologous
Non-Hodgkin's Lymphoma	10	Nine allogeneic except for one autologous
Chronic Myeloid Leukaemia	1	Allogeneic
Acute Lymphoblastic Leukaemia	1	Allogeneic
Severe Aplastic Anaemia	1	Allogeneic
Myelodysplastic Syndromes	1	Allogeneic

Data represents the number of patients, their diagnosis and the type of bone marrow grafts they received.

The preconditioning of patients for transplantation included 120 mg/kg of cyclophosphamide (Cy), 12 Gy of fractionated total body irradiation (TBI) and 6 Gy of total nodal irradiation (TNI). Bone marrow from patients was aspirated under intramuscular pethidine HCL (1 mg/kg of body weight) analgesia given 30 minutes prior to the local anaesthetic administration.

5.2 Methods

For mononuclear cells preparation, refer to chapter 2 for a description of the method.

5.2.1 Stromal Assay

This assay was performed to evaluate the effects of bFGF on patients' stromal layer development in the recovery stage following transplantation and compared to stromal layers from normal samples. For the concentration and conditions used for the establishment of stromal layers, refer to chapter 2 for a description of the method. Stromal layers were cultured in the absence and presence of the 2 and 20 ng/ml bFGF with 20 ng/ml heparan sulphate.

5.2.2 Fibroblastic Progenitor Assay

This assay was performed to measure the effects of bFGF on the fibroblastic progenitors from patients receiving transplantation and compared to those from normal individuals. Refer to chapter 4 for a description of the method. Fibroblastic colonies from patients were cultured in the absence and presence of 2 and 20 ng/ml bFGF with 20 ng/ml heparan sulphate.

5.2.3 Progenitor Cell Selection

Refer to chapter 2 for a description of the method for the selection of $CD34^+$ cells. Aliqouts of selected cells from patients were studied by standard flow-cytometry.

5.2.4 Blastic Colony Assay

This assay was carried out to assess the effects of bFGF on the ability of patients' CD 34^+ cells to proliferate and form blastic colonies on normal preformed stroma. Furthermore, the effects of bFGF on the impaired stromal supportive capacity, was evaluated. Normal $CD34^+$ cells were also cultured on normal stroma in order to provide comparative results. Blastic colony assay was performed in the absence and presence of the 2 and 20 ng/ml bFGF with 20 ng/ml heparan sulphate. Refer to chapter 2 for a description of the method.

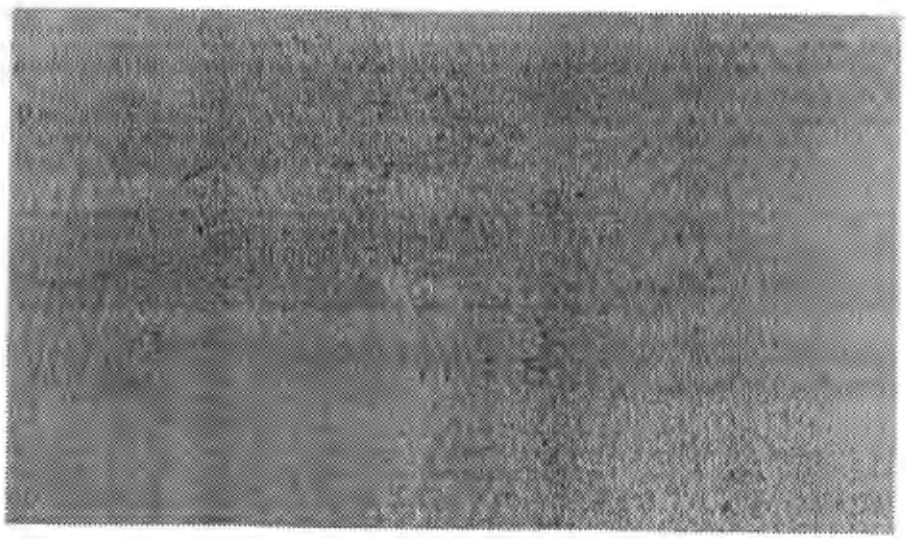
5.3 Statistical Analysis

Statistical analysis was performed by both the student t-test and paired test, comparing normal cells and patients' cells cultured in the presence of bFGF. Significance level was set at <0.05.

5.4 Results

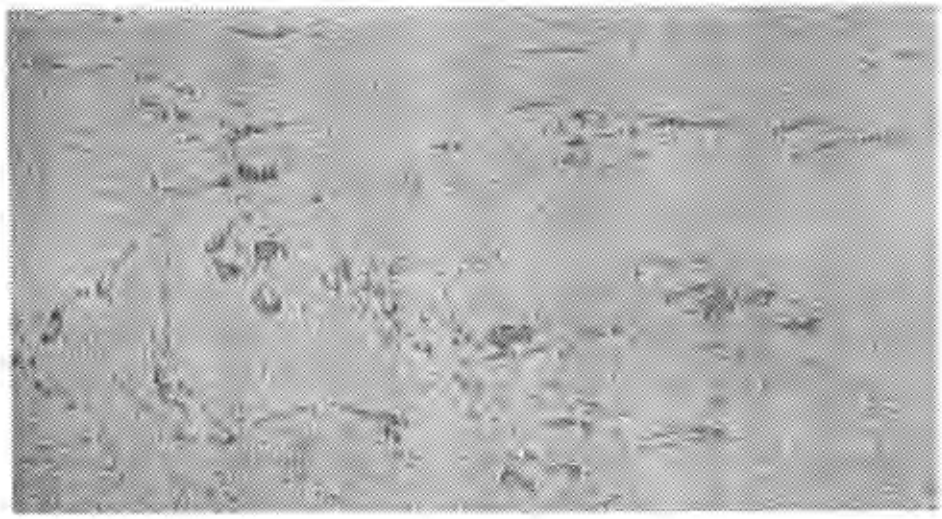
5.4.1 Appearance of Bone Marrow Stroma

Picture 5A



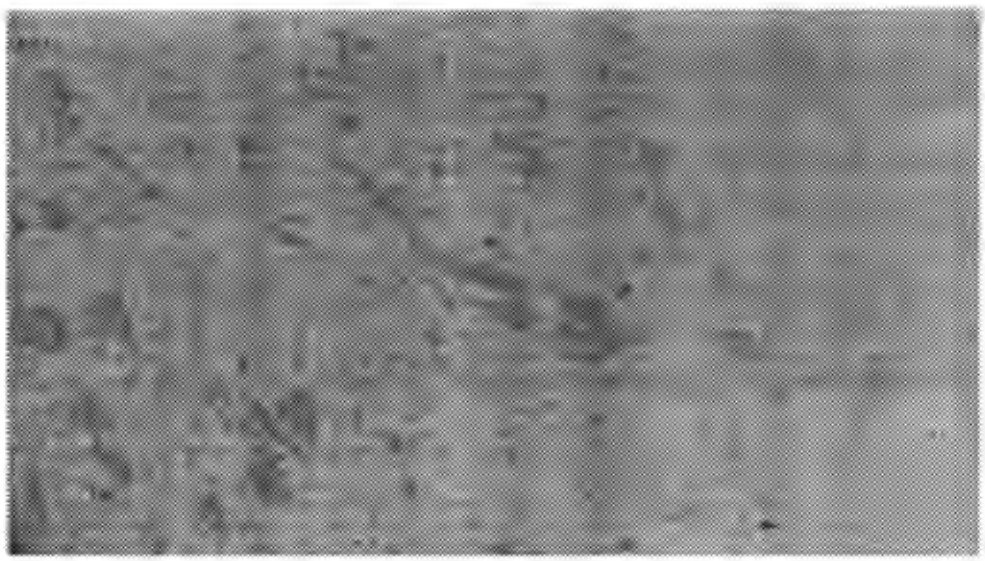
Picture 5A. *Stromal layer from a patient cultured at 0.5 x10^6/ml mononuclear cells for 3 weeks in the absence of bFGF.*

Picture 5B



Picture 5B. *Stromal layer from a normal individual cultured at 0.5 x10^6/ml mononuclear cells for 3 weeks in the absence of bFGF. This stromal layer is patchy, as one could visualize a band where stroma is covering the surface area of the dish and the other parts of the dishes where there is no stroma at all.*

Picture 5C



Picture 5C. *Stromal layer from a patient cultured at 0.5 x10^6/ml mononuclear cells for 3 weeks in the presence bFGF. One could visualize large globules of fat cells and stroma is covering almost the whole surface of the dish. There are lots of macrophages in the stroma.*

Results show that stromal layers cultured in the presence of bFGF grew in multilayers. It was possible to visualize different directions of the growing fibroblasts. This improvement in stromal layers could match stromal layers from normal individuals cultured in the absence of bFGF (Pictures 5C vs. 5A). Fat cells in bFGF-supplemented cultures appeared increased in size as a result of their larger globules. Macrophages in bFGF-treated cultures also appeared increased in numbers when compared to those in control culture dishes and normal individuals (Pictures 5C vs. 5A and 5C).

Table 5.2. The effect of bFGF on the growth of patients' stromal cells

Culture	Median Confluence (%)	Range	Comparison between normal, 2 and 20 ng bFGF
Normal	50	40-65	
Control	40	0-55	0.0001
2 ng/ml bFGF	62.5	0-88	0.47
20 ng/ml bFGF	78	20-98	0.001

0.5 x 10^6/ml cells were cultured in the absence and presence of 2 and 20 ng bFGF with 20 ng/ml heparan sulphate, in α-MEM supplemented with 15% of each FCS, horse serum and 2 x 10^{-7} M HC. They were incubated at 37°C in 5% CO_2 for 21 days. The last column gives a comparison between the area covered in normal individuals and in patients in the absence and presence bFGF concentrations. Significance level was set at <0.05.

Mononuclear cells from patients undergoing transplantation were cultured in 35 mm petri dishes for 3 weeks, in the presence of 0, 2 and 20 ng/ml bFGF with 20 ng/ml heparan sulphate. The median surface area covered by stromal layers in normal and patients' samples was 50 and 40% respectively. The surface area improved upon the addition of 2 ng/ml bFGF (62.5 vs. normal 50%; p=0.47), and significantly increased with the addition of 20 ng/ml bFGF (78 vs. normal 50%; p=0.001; Table 5.2).

5.4.2 Stromal Cell Numbers

Table 5.3. The effect of bFGF on the proliferation of patients' stromal cells

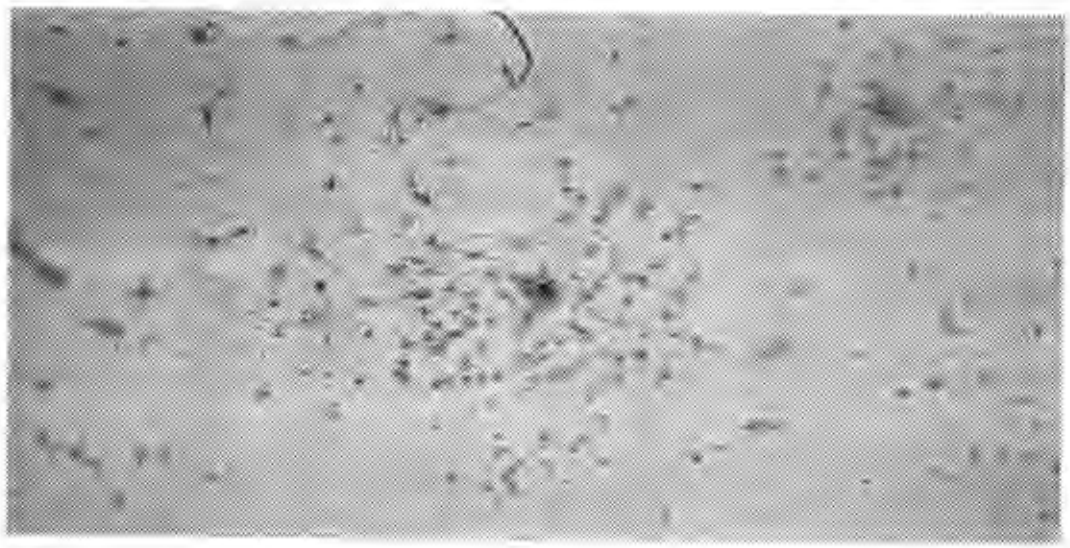
Culture	Cell Numbers x 10^6/2ml (Median)	Range	Comparison between normal, 2 and 20 ng bFGF
Normal	0.61	0.15-1.66	
Control	0.66	0.28-1.73	0.1
2 ng/ml bFGF	1.44	0.25-2.8	0.003
20 ng/ml bFGF	2.11	0.46-3.4	<0.05

MNC cultured for 21 days were quantitated by addition of 5% trypsin solution to stromal dishes for 10 minutes. Single cell suspensions were washed off the dishes and stromal cells counted with a particle counter. The paired t-test gives a comparison between normal, 2 and 20 ng/ml bFGF and 20 ng/ml heparan sulphate. Significance level was set at <0.05.

Single cell suspensions from trypsinized stromal layers supplemented with 0, 2 and 20 ng/ml bFGF were prepared in order to determine the effects of bFGF on stroma. Monolayers established with 0.5 x 10^6/ml mononuclear cells in patients produced 0.66 x 10^6/2ml cells (range 0.15-1.66 vs. normal 0.61) and they increased significantly with the addition of 2 and 20 ng/ml bFGF with 20 ng/ml heparan sulphate ($p=0.003$ and <0.05; Table 5.3).

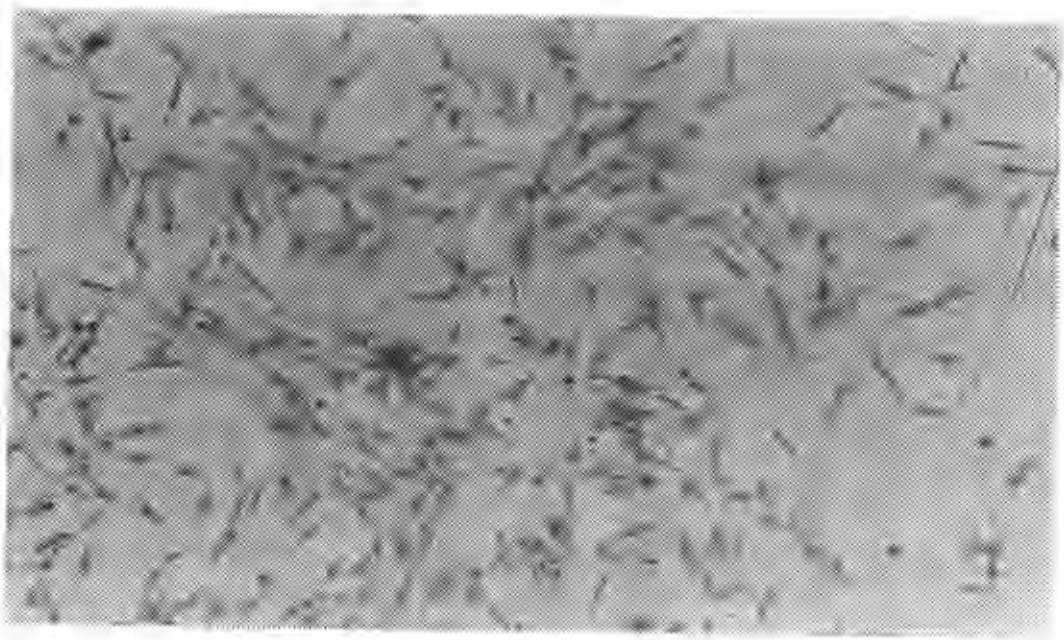
5.4.3 Fibroblastic Progenitors

Picture 5D



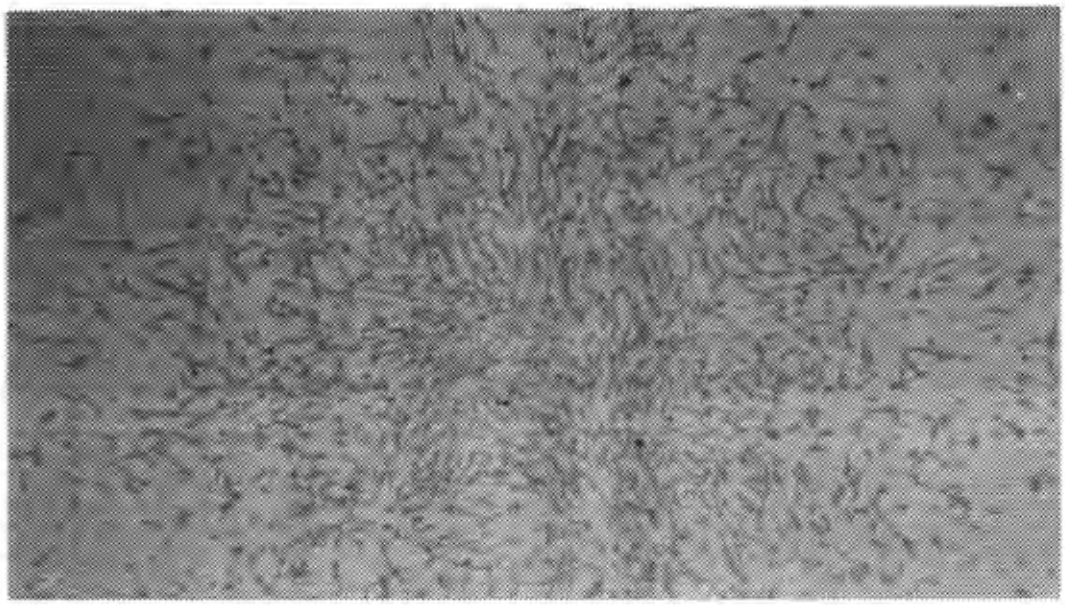
Picture 5C. *Fibroblastic progenitor from a patient cultured at 1 x 10^6/5ml for 9 days in the absence of bFGF and stained with MGG.*

Picture 5E



Picture 5E. *Fibroblastic progenitor from a normal individual cultured at 1 x 10^6/5ml for 9 days in the absence of bFGF and stained with MGG. Colonies (like the one in the center) are discrete and easily counted.*

Picture 5F



Picture 5F. *Fibroblastic progenitor from a patient cultured at 1 x 10^6/5ml for 9 days in the presence of bFGF and stained with MGG. Picture shows many colonies that have grown towards each other, therefore forming one large colony.*

Patients' fibroblastic progenitors cultured in the presence of bFGF occupy almost the whole surface area of the dish, in such a way that colonies were difficult to quantitate. Fibroblastic colonies from normal individuals and controls cultured in the absence of bFGF were discrete and easily quantitated (Pictures 5F vs. 5D and 5E).

Table 5.4. The effect of bFGF on growth of patients' fibroblastic progenitors

Control	CFU-F x 10^6/5ml (Median)	Range	Comparison between normal, 2 and 20 ng bFGF
Normal	28.3	14-53	
Control	7	5-28	0.0001
2 ng/ml bFGF	10	3-33	0.001
20 ng/ml bFGF	9	4-33	0.002

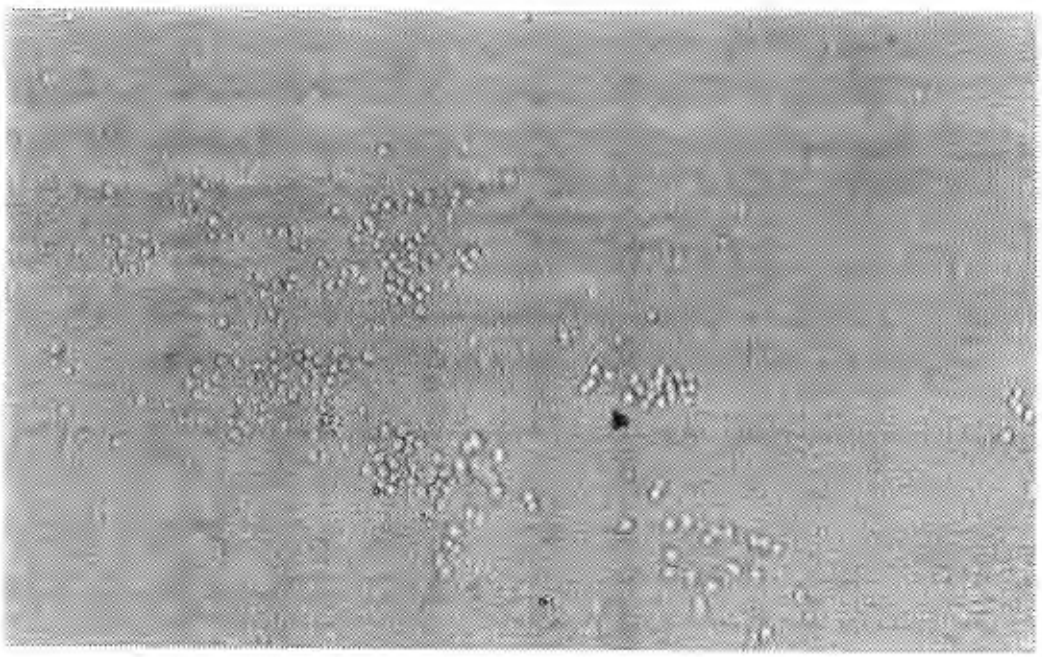
MNCs were cultured in α-MEM supplemented with 20% FCS for 9 days. The last column compares the normal, 2 and 20 ng/ml bFGF and 20 ng/ml heparan sulphate. Statistical analysis was given by the paired t-test and significance level was set at < 0.05.

Following 9 days of incubation, fibroblastic colony cultures were terminated, fixed, stained with MGG and colonies scored using an inverted microscope. The median fibroblastic colony numbers in normal individuals were 28.3 x 10^6/5ml (range 14-53), and these values increased above those in control samples (median 28.3 vs. 7 x 10^6/5ml; p=0.0001). The addition of 2 and 20 ng/ml bFGF with 20 ng/ml heparan sulphate did not change the colony numbers (10 and 9 vs. 28.3; Table 5.4).

5.4.4 Blastic colonies

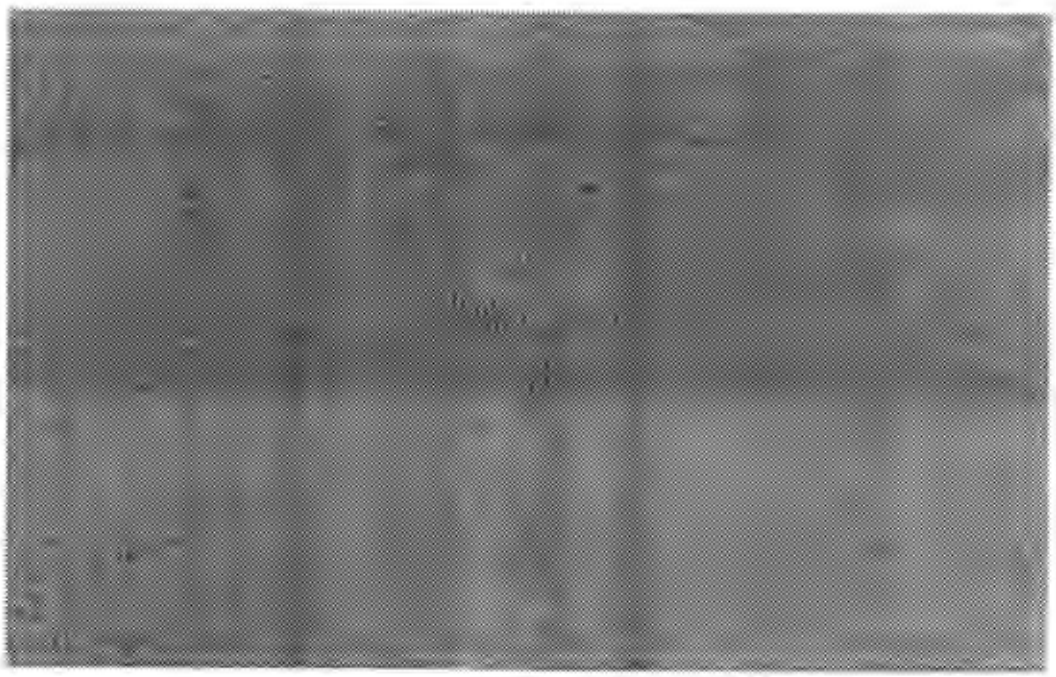
Normal or patients' selected cells expressing $CD34^+$ antigen was 92.4 or 89.4% of the $CD34^+$ population respectively. Selected $CD34^+$ cells were layered in duplicate in cross-culture studies on normal or patients' preformed stroma. Normal $CD34^+$ cells were also cultured on normal stroma in order to provide comparative results.

Picture 5G



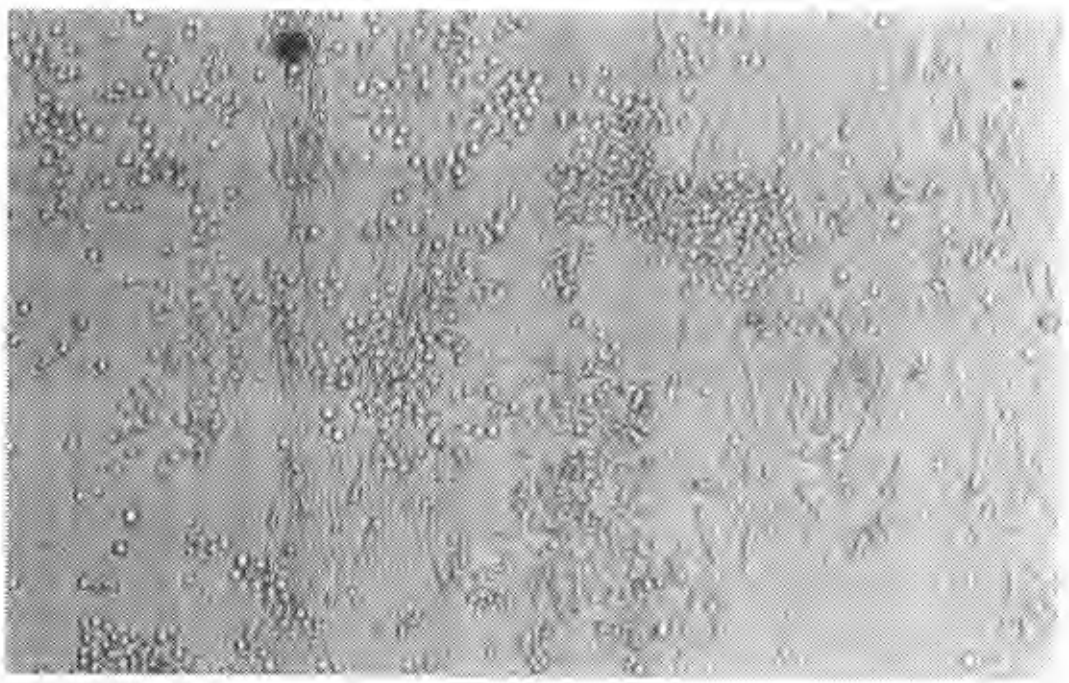
Picture 5G. *Blastic colonies obtained when normal 1 x 10^4/ml $CD34^+$ cells were layered on patients' stroma in the absence of bFGF.*

Picture 5H



Picture 5H. *Blastic colonies obtained when normal 1 x 10^4/ml $CD34^+$ cells were panned on normal stroma in the absence of bFGF.*

Picture 5I



Picture 5I. *Blastic colonies obtained when normal 1 x 10^4/ml $CD34^+$ cells were cultured on patients' stroma in the presence of bFGF.*

Blastic colonies from patients cultured in the presence of bFGF were larger, matching those blastic colonies obtained from normal individuals in the absence of bFGF (Pictures 5I vs. 5H). One could visualize that patients' stroma-adherent cells making up a blastic colony, in the absence of bFGF, are small and tend to be loose from each other (Picture 5G). On the other hand, stroma-adherent cells from a patient cultured in the presence of bFGF formed firm and discrete colonies (Pictures 5G vs. 5I).

Table 5.5. Effect of bFGF on the proliferation of patients' $CD34^+$ cells on normal preformed stroma compared to proliferation of normal $CD34^+$ cells on normal stroma

Control	CFU-bl x 10^4/ml (Median)	Range	Comparison between normal, 2 and 20 ng bFGF (P=Value)
Normal	90	24-139	
Control	D6=40	3-121	0.009
	D9=37	5-130	
	D11=36	5-215	
2 ng/ml bFGF	D6=64.5	6-193	0.3
	D9=70.5	6-201	
	D11=51	24-331	
20 ng/ml bFGF	D6=85	5-228	0.7
	D9=96	6-255	
	D11=80	13-423	

Results of blastic colonies when patients cells were layered on normal stroma which was treated with 2 and 20 ng bFGF and cultured for 6, 9 and 11 days. The second row represents blastic colonies obtained when normal $CD34^+$ cells were layered on to normal stroma, covered with 0.3% agar and incubated for 6 days. The last column compares the normal, 2 and 20 ng/ml bFGF with 20 ng/ml heparan sulphate. Statistical analysis was given by the paired t-test and significance level was set at <0.05

The median number of CFU-bl when normal 1 x 10^4/ml $CD34^+$ cells were cultured on normal stroma was 90 x 10^4/ml (range 24-139). Blastic colony numbers in patients were significantly lower (median 40 x 10^4/ml; range 3-121; p=0.009), but improved upon the addition of 2 and 20 ng/ml bFGF, matching blastic colony scores from normal individuals (64.5 and 85 vs. normal 90 x 10^4/ml; Table 5.5).

Table 5.6. Effect of bFGF on the proliferation of normal $CD34^+$ cells on patients' preformed stroma and compared to the proliferation of normal $CD34^+$ cells on normal stroma.

Control	CFU-bl x 10^4/ml (Median)	Range	Comparison between normal, 2 and 20 ng bFGF (P-Value)
Normal	90	24-139	
Control	D6=39	6-84	0.001
	D9=37	6-90	
	D11=26.5	3-139	
2 ng/ml bFGF	D6=60	11-111	0.05
	D9=61	7-130	
	D1145	2-89	
20 ng/ml bFGF	D6=91	6-133	0.2
	D9=92	9-141	
	D11=71	7-139	

Results of blastic colonies when normal $CD34^+$ cells were cultured for 6, 9 and 11 days, on patients' stroma which was treated with 2 and 20 ng bFGF. The second row represents blastic colonies obtained when normal $CD34^+$ cells were layered on to normal stroma, covered with 0.3% agar and incubated for 6 days. The last column compares the normal, 2 and 20 ng/ml bFGF with 20 ng/ml heparan sulphate. Statistical analysis was given by the paired t-test and significance level was set at <0.05.

The median number of CFU-bl when normal $CD34^+$ cells were layered on normal stroma was 90 x 10^4/ml (range 24-139). Blastic colony scores when normal $CD34^+$ cell were panned on patients' stroma was 39 x 10^4/ml (range 6-84), but improved significantly upon the addition of 2 ng/ml bFGF (60 x 10^4/ml CFU-bl; p=0.05). The addition of 20 ng/ml bFGF gave blastic colony scores which were higher than those in control samples (91 vs. 39 x 10^4/ml CFU-bl), and matching colony scores from normal individuals (91 vs. normal 90 CFU-bl; Table 5.6)

5.5 Discussion

In vitro, abnormalities that follow recovery from stem cell transplantation include: failure of the stromal layer to cover the whole surface area of the dish, and poor stromal support to the growth of blastic colonies.

Data demonstrates that stromal layers formed by patients' cells cultured in the absence of bFGF cover a lesser area than the area covered by stromal layers in normal individuals and this finding supports the observation found in bone marrow transplantation by Novitzky et al, 1995 [70]. Patients' cells cultured in the presence of bFGF occupy a greater surface area than the area covered by stromal layers in normal individuals and controls. The other finding is that bFGF accelerates the proliferation rate of the stromal cells, since stromal layers in its presence become confluent within 3 weeks, while stromal layers cultured in the absence of bFGF initiated at the same time, are still irregular.

Basic FGF also improves the appearance of fat cells and fibroblast in patients' stromal cultures, providing a better background and environment for haematopoietic cells to survive, proliferate and differentiate.

Results also show that patients' stromal cell numbers in the presence of bFGF are significantly increased above those cell numbers in controls and normal individuals. Augmentation in patients' cell numbers in the presence of bFGF corresponds to the results of surface area covered by stroma, since surface area covered in the presence of bFGF is also increased above that in normal individuals. Patients' cell numbers cultured in the absence of bFGF are increased above cell numbers from normal individuals, but are not significantly different from each other.

Basic FGF stimulates the growth of fibroblasts and this is supported by the fact that colonies from patients' samples, in its presence, are larger and growing towards each other as compared to colonies in control samples (which are small, discrete and easily counted). These results show that bFGF might not have an incremental effect on the number of fibroblastic progenitors, but it does increase the sizes of the colonies.

Results also demonstrate that bFGF significantly elevates the clonogenic capacity of patients' cells only when used at the highest concentration, which is the 20 ng/ml. This increase in patients' blastic colonies match blastic colony scores obtained when normal cells are cultured on normal stroma. This means that patients' $CD34^+$ cell cultured in the presence of 20 ng/ml bFGF have similar clonogenic potential as normal $CD34^+$ cells cultured in the absence of bFGF.

Basic FGF also improves the integrity of patients' stroma since it supports the growth normal blastic colonies better. This statement is supported by the fact that colony numbers obtained on patients' stroma are the same as the colony scores found when normal $CD34^+$ are layered on to normal stroma. This means that patients' stroma in the presence of bFGF supports normal blastic colonies in the same manner as normal stroma in the absence of bFGF.

For a comparative analysis, data was inadequate to illustrate similarities or differences in patients receiving allogeneic and autologous grafts, since there were more allogeneic patients (n=13) than those receiving autologous (n=3). The difference between patients studied at 6 and 12 months was also not evaluated since more patients were studied at 6 months (n=11) and few at 12 months (n=5).

5.6 Conclusion

Patients' stromal cells in the presence of bFGF proliferate faster than normal stromal cells. It was further illustrated that bFGF does not augment patients' fibroblastic colony numbers, but only increases the sizes of the colony. It improves the clonogenic capacity of patients' cells, which is associated with greater proliferation of patients' $CD34^+$ leading to increased number of blastic colonies. The integrity of patients' stroma is also improved in that more cells adhere to stroma, leading to an increased proliferation of those adhered cells.

In conclusion, basic Fibroblastic Growth Factor has the following *in vitro* effects on patients who had undergone peripheral stem cell transplantation:

- Improves the proliferation rate of patients' stromal layers above that of a normal population.
- Significantly elevates patients' stromal cell numbers above normal stromal cell numbers.
- Unable to increase fibroblastic colony numbers above those in normal individuals.
- Improves the integrity of patients' stroma for the growth of normal blastic colonies.

CHAPTER 6

Overall Summary

Normal haematopoiesis is characterized by the balanced interplay between haematopoietic stem/progenitor cells, molecules and cells that form part of the microenvironment. Cell adhesion molecules (CAMs) expressed on the cell surfaces of the haematopoietic stem/progenitor cells bind to specific ligands on the stromal cells and the extracellular matrix (ECM) components.

This interaction is important in that haematopoietic cells will be in contact with the growth factors produced by the stromal components. Heparan sulphate proteoglycans (HSPGs) also play an important role in haematopoiesis because they bind to growth factors and present them in an active form to the haematopoietic cells within the microenvironment. This haematopoietic microenvironment is a highly organized structure where stem cells interact closely with the marrow stroma, and any alterations in the microenvironment may result in abnormal production of blood cells.

In an attempt to eradicate these malignancies, bone marrow or peripheral blood stem cell transplantation is employed. Abnormal cells are replaced with bone marrow stem cells derived from either another individual or with a previously harvested portion of the patients' own marrow. Patients who participated in this study were in complete remission of their haematological malignancies at 6 or 12 months post-transplantation. Bone marrow recipients in this study were of either related or unrelated stem cell grafts.

It was described by Novitzky et al, (1995) that following autologous or allogeneic transplantation, derangement in the proliferation and function of the bone marrow stroma and of selected $CD34^+$ cells was noted. It was shown by the poor stromal supportive capacity and a reduction in the clonogeneic potential of the stroma adherent $CD34^+$ cells, which did not recover even 8 years after transplantation.

In a further study, the use of blood stem cells for transplantation showed a more favourable development of the stroma. This improved proliferation of stromal elements and support for the multiplication of $CD34^+$ cells suggests that a large progenitor cell pool or harvest of elements that may be important for the formation of the stroma lead to a partially corrected haematopoiesis although the origin of the stroma was not tested. Given the above defects, basic fibroblast growth factor (bFGF) is considered to be a potentially useful cytokine to induce correction of these abnormalities.

This growth factor was chosen, as it was shown to be a multifunctional cytokine that accelerates the *in vitro* formation of the stromal cell layer and increases the number of adherent and non-adherent cells in the stroma.

Basic FGF was used to supplement medium for culturing post-transplantation stromal layers. In patients, bFGF induced stroma to cover the entire surface growth area by increasing the number of cells in the stromal layer, so that when haematopoietic cells are generated they can survive and proliferate in close contact with this stromal layer. This means that bFGF restores the damaged stromal components and provides a suitable environment for the haematopoietic progenitor cell survival.

Fibroblastic colonies in bFGF-untreated cultures are discrete and their numbers are more accurately enumerated than those colonies in bFGF-supplemented cultures. During our study period, colonies in bFGF-supplemented cultures covered the whole surface area of the dish to the extent that they were difficult to count and this is a limitation of this study, as it is based mainly on quantitative measurements.

Basic FGF increases this clonogeneic potential $CD34^+$ cells onto preformed stroma leading to the proliferation of more cells. In this study it is noted that in the presence of bFGF, blastic colony numbers from patient are increased to a greater extent than colony numbers from the same patients in the absence of bFGF.

Data demonstrate that bFGF improves the impaired patients' stroma, to be a more suitable, better environment for the growth, proliferation and differentiation of the haematopoietic cells. Stromal cells in its presence are able to cover the available growth surface area, thereby releasing the necessary growth factors needed for specific effects on the haematopoietic stem cells and in this way haematopoiesis is improved.

Future Intentions

The future direction of this study will be to look at the *in vivo* effects of bFGF in animals. A murine FGF will be obtained and employed in the animal study to assess the morphological effects of this growth factor and comparing them with the *in vitro* results obtained. Provided this protein is not toxic to animals, animals will undergo transplantation and the cytokine will be used *in vivo*. If similar *in vitro* abnormalities corrected in humans are rectified in animals, then it may justify the use of bFGF in patients.

REFERENCES

1. Abrahamsen JF, Smaaland R, Sothern RB, Laerum OD, **"Variation in Cell Yield and Proliferative Activity of Positive Selected Human $CD34^+$ Bone Marrow Cells along the Circadian Time Scale",** European Journal of Haematology 1998: 60: pages 7-15.
2. Alessandro A, Christof F, Colin AS, et al, "**Identification of Distinct Elements of the Stromal Microenvironment That Control Human Haematopoietic Stem/Progenitor Cell Growth and Differentiation",** Experimental Haematology 1998: 26: pages 143-157.
3. Arakawa T, Wen J, Philo JS, **"Stoichometry of Heparin Binding to basic Fibroblast Growth Factor",** Archives of Biochemistry and Biophysics 1994: 308: pages 267-273.
4. Ashihara E, Shimazaki C, Sudo Y, et al, **"FLT-3 Ligand Mobilizes Haematopoietic Primitive and Committed Progenitor Cells into Blood in Mice",** European Journal of Haematology 1998: 60: pages 86-92.
5. Asosingh K, Renmans W, Van der Gucht, et al, **"Circulating $CD34^+$ Cells in Cord Blood and Mobilized Blood Have a Different Profile Adhesion Molecules Than Bone Marrow $CD34^+$ Cells",** European Journal of Haematology 1998: 60: pages 153-160.
6. Arnold R, Schmeiser T, Helt W, "**Haematopoietic Cell Reconstitution after Human Marrow Transplantation",** Transplant Procedures 1986: 14: page 271.
7. Arnon N, Rosales F, Reuven O, et al, **"The Role of Thiotepa in Autologous Bone Marrow Transplantation For Acute Leukaemia",** Leukaemia Research 1998: 22; pages 991-995.
8. Bacigalupo A, Panagiotis Z, Van Lint M, et al, **"Allogeneic Bone Marrow or Peripheral Blood Cell Transplants in Adults With Haematological Malignancies: A Single-Centre Experience",** Experimental Haematology 1998: 26: pages 409-414.
9. Baines P, Masters G, Booth M, et al, **"Enrichment of Progenitor Cells From Human Marrow",** Experimental Haematology 1987:
10. Berardi A, Wang A, Abraham J, et al, **"Basic Fibroblastic Growth Factor Mediates its Effects on Committed Myeloid Progenitors by Direct Action and Has no Effect on Haematopoietic Stem Cells",** Blood 1995: 86: pages 2123-2129.
11. Blair A, Thomas DB, **"Preferential Adhesion of Fetal Liver Derived Primitive Haematopoietic Progenitor Cells in the Bone Marrow Stroma",** British Journal of Haematology 1997: 99:
    pages 726-731.

12. Bodine DM, Seidel NE, Orlin D, **"Bone Marrow Collected 14 Days After in Vivo Administration of Granulocyte-Colony Stimulating Factor and Stem Cell Factor to Mice Has 10-fold More Repopulating Ability Than Untreated Bone Marrow",** Blood 1996: 88: pages 89-97.
13. Bot FJ, Van Eijk L, Scchipper P, et al, **"Synergistic Effects Between GM-CSF and G-CSF or M-CSF on Highly Enriched Marrow Progenitor Cells",** Leukaemia 1990: 4: pages 325-328.
14. Breems DA, Blokland AW, Siebel KE, et al, **"Stroma-Contact Prevents Loss of Haematopoietic Stem Cell Quality During Ex Vivo Expansion of $CD34^+$ Mobilized Blood Stem Cells",** American Society of Haematology 1998: pages 111-116.
15. Brunner G, Nguyen H, Gabrilove J, et al, **"Basic Fibroblast Growth Factor Expression in Human Bone Marrow and Peripheral Blood Cells",** Blood 1993: 81: pages 631-638.
16. Brunner G, Metz CN, Nguyen H, et al, **"An Endogenous Glycosylphoshatidyl-Specific Phospholipas-D Releases Basic Fibroblast Growth Factor-Heparan Proteoglycan Complexes From Human Bone Marrow Cultures",** Blood 1994: 83: pages 2115-2125.
17. Bruno E, Cooper RJ, Wilson EL, et al, **"Basic Fibroblast Growth Factor Promotes the Proliferation of Human Megakaryocyte Progenitor Cells",** Blood 1993: 82: pages 430-435.
18. Burger PE, Dowdle EB, Lukey PT, et al, **"Basic Fibroblast Growth Factor Antagonizes Transforming Growth Factor-β Mediated Erythroid Differentiation in K562 Cells",** Blood 1994: 83: pages 1808-1812.
19. Bunjes D, Theobald M. Hertenstein B, et al, **"Successful Therapy With Donor Buffy Coat Transfusions in Patients With Relapsed Chronic Myeloid Leukaemia After Bone Marrow Transplantation is Associated With High Frequencies of Host-Reactive Interleukin-2 Secreting T-Helper Cells",** Bone Marrow Transplantation 1995: 15: pages 713-719.
20. Chabannon C, Storb T, **"Stem Cell-Stromal Cell Interactions",** Current Topic in Microbiology and Immunology 1992: 177: 123-128.
21. Chiang KY, Weisdorf DJ, Davies SM, et al, **"Outcome of Second Bone Marrow Transplantation Following a Uniform Conditioning Regimen as Therapy For Malignant Relapse",** Bone Marrow Transplantation 1996: 17: pages 39-42.
22. Clarke E, McCann SR, **"Stromal Colonies Can Be Grown From The Non-Adherent Cells in Human Long-Term Bone Marrow Cultures",** European Journal of Haematology 1991: 46: pages 296-300.
23. Dale DC, Liles WC, Llewellyn C, et al, **"Effects of Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) on Neutrophil Kinetics and Function in Normal Human Volunteers",** American Journal of Haematology 1998: 57: pages 7-15.

24. De Waele M, Renmans W, Damiaens S, et al, **"Different Expression of Adhesion Molecules On Myeloid and B-Lymphoid CD34$^+$ Progenitors in Normal Bone Marrow,"** European Journal of Haematology 1997: 59: pages 277-286.

25. De Waele M, Renmans W, Jochmans K, et al, **"Different Expression of Adhesion Molecules on CD34$^+$ Cells in AML and B-lineage ALL and Their Normal Bone Marrow Counterparts",** European Journal of Haematology 1999: 63; pages 192-201.

26. Dexter TM, Moore MA, **"In Vitro Duplication and "Cure" of Haematopoietic Defects in Genetically Anaemic Mice",** Nature 1977:269: page 412.

27. Dexter TM, Heyworth, **"Growth Factors and The Molecular Control of Haematopoiesis",** European Journal of Clinical Microbiology and Infectious Diseases 1994: 13: pages 3-8.

28. Dimitry AB, Ellen AWB, Karen ES, et al, **"Stroma-Contact Prevents Loss of Haematopoietic Stem Cell Quality During Ex-Vivo Expansion of CD34$^+$ Mobilized Peripheral Blood Stem Cells",** American Society of Haematology 1998: pages 111-116.

29. Domenech J, Gihana E, Dayan A, et al, **"Haematopoiesis in Transplanted Patients With Autologous Marrow Associated By Long-Term Marrow Culture",** British Haematology 1994: 88: pages 488.

30. Donnelly DS, Zelterman D, Sharkis S, et al, **"Function Activity of Murine CD34$^+$ and CD34$^-$ Haematopoietic Stem Cell Populations",** Experimental Haematology 1999: 27: pages 788-796.

31. Dowding CR, Gordon MY, **"Physical, Phenotypic and Cytochemical Characteristics of Stroma -Adherent Blast Colony Forming Cells",** Leukaemia 1992: 6: pages 347-351.

32. Eaves C, Cashman J, Eaves A, **"Defective Regulation of Leukaemia Haematopoiesis in Chronic Myeloid Leukaemia",** Leukaemia Research 1998: 22: pages 1085-1096.

33. Freisel RE, Maclag T, **"Molecular Mechanisms of Angiogenesis: Fibroblast Growth Factor Signal Transduction",** FASEB Journal 9: pages 919-925.

34. Gabrilove JL, White K, Rahman Z, et al, **"Stem Cell Factor and Basic Fibroblast Growth Factor are Synergistic in Augmenting Committed Myeloid Progenitor Cell Growth",** Blood 1994: 83: pages 907-910.

35. Gruber G, Schwarzmeier JD, Shehata M, et al, **"Basic Fibroblast Growth Factor is ExpressedBy CD19/CD11c-Positive Cells in Hairy Cell Leukaemia",** Blood 1999: 94: pages 1077-1085.

36. Goliaei B, Soheili Z, Behboodi A, et al, **"Haematopoiesis in The Presence of Macrophages in Long–Term Bone Marrow Cultures",** Experimental Haematology 1995: 23: pages 1115-1120.

37. Gordon MY, Clarke D, Artkins J, et al, **"Haematopoietic Progenitor Cell Binding to the Stromal Microenvironment In Vitro",** Experimental Haematology 1990: 18: pages 837-842.

38. Gordon MY, Dowding CR, Riley GP, et al, **"Characterization of Stroma-Dependent Blast Colony-Forming Cells in Human Marrow"**, Journal of Cellular Physiology 1987: 130: pages 150-156.

39. Gordon MY, Lewis JL, Marley SB, et al, **"Stromal Cells Negatively Regulate Primitive Haematopoietic Progenitor Cell Activation Via a Phosphotidylinositol-Anchored Cell Adhesion/Signaling Mechanism"**, British Journal of Haematology 1997: 96: pages 647-753.

40. Grontos S, Simmons P, **"The Growth Factor Requirements of Stro-1-Positive Human Bone Marrow Stromal Precursor under Serum Deprived Conditions In Vitro"**, Blood 1995: 85: pages 929-940.

41. Gross S, Helm K, Gruntmeir JJ, et al, **"Characterization and Phenotypic Analysis of Differentiating $CD34^+$ Human Bone Marrow Cells in Liquid Culture"**, European Journal of Haematology 1997: 59: pages 318-326.

42. Hannocks M, Olivier L, Gabrilove JL, et al, **"Regulation of Proteolytic Activity in Human Bone Marrow Stromal Cells By Basic Fibroblast Growth Factor, Interleukin-1 and Transforming Growth Factor-β"**, Blood 1992: 79: pages 1178-1184.

43. Harvey DP, Stuart K, **"A Proposal Regarding the Mechanism which Underlies Lineage Choice During Haematopoietic Differentiation"**, Leukaemia Research 1999: 23: pages 685-694.

44. Hockland P, Carlsen I, Hokland M, et al, **"Modulatory Function on Autologous Myeloid Progenitor Cells of Clonal T-Lymphocytes Following Bone Marrow Transplantation"**, European Journal of Haematology 1998: 60: pages 189-196.

45. Huang S, Chen Z, Feng J, et al, **"Correlation Between IL-3 Receptor Expression and Growth Potential of Human $CD34^+$ Haematopoietic Cells From Different Tissues"**, Stem Cells 1999: 17: pages 265-272.

46. Ketterer N, Salles G, Raba M, et al, **"High $CD34^+$ Count Decreases Haematologic Toxicity of Autologous Peripheral Blood Progenitor Cell Transplantation"**, Blood 1998: 91: pages 3148-3155.

47. Koya T, Narita J, Honda S, et al, **"Erythropoietin Induces the Expansion of c-Kit^+ Progenitor For Myeloid and Erythroid Cells, But Not for Lymphoid Cells, in the Bone Marrow and Liver"**, European Journal of Haematology 1999: 63: pages 306-312.

48. Krause DS, Kapadia SU, Raj NB, et al, **"Regulation of CD34 Expression in Differentiating M1 cells",** Experimental Haematology 1997: 25: pages 1051-1061.

49. Krause DS, Fackler MJ, Civin CI, et al, **"CD34: Structure, Biology and Clinical Utility",** Blood 1996: 87: pages 1-13.

50. Laurie EA, Brigitte G, Donna EH, et al, **"Detection and Characterization of Primitive Malignant and Normal Progenitors in Patients with Acute Myelogenous Leukaemia using Long-Term Coculture with Supportive Feeder Layers and Cytokines",** Blood 1997: 90: pages 2555-2564.

51. Lee J, Kook H, Chung I, Kim H, Park M, **"Telomere Length Changes in Patients Undergoing Haematopoiesis Stem Cell Transplantation",** Bone Marrow Transplantation 1999: 24: pages 411-415.

52. Liesveld JL, Winslow JM, Kempski MC, et al, **"Adhesive Interactions of Normal and Leukaemia Human CD34$^+$ Myeloid Progenitors: Role of Marrow Stromal, Fibroblast, and Cytomatrix Components"**, Experimental Haematology 1991: 19: pages 63-70.

53. Li J, Sensebe L, Herve P, et al, **"Non-Transformed Colony-Derived Stromal Cell Lines From Normal Human Marrow: Phenotypic Characterization and Differentiation Pathway",** Experimental Haematology 1995: 23: pages 133-141.

54. Maciejewski JP, Selleri C, Sato T, et al, **"A Severe and Consistent Deficit in Marrow Circulating Primitive Haematopoietic Cells (LTC-IC) in Acquired Aplastic Anaemia",** Blood 1996: 88: pages 1983-1991.

55. Manabe A, Coustan-Smith E, Behm FG, et al, **"Bone Marrow Derived Stromal Cells Prevent Apoptotic Death in B-Lineage Acute Lymphoblastic Leukaemia",** Blood 1992: 79: pages 2370-2377.

56. Masahiro O, Yoshikazu K, Osamu I, et al, "**Comparative Multi-Colour Flow Cytometric Analysis of Cell Surface Antigens in Bone Marrow Haematopoietic Progenitors Between Refractory Anaemia and Aplastic Anaemia",** Leukaemia Research 2000: 24: pages 359-366.

57. Masek LC, Sweetenham JW, Whitehouse MA, **"Immuno-, Lectin-, and Enzyme-Histochemical Characterization of Human Bone Marrow Endothelium",** Experimental Haematology 1994: 22; pages 1203-1209.

58. Majors A, Ehrhart LA, **"Basic Fibroblast Growth Factor in the Extracellular Matrix Suppresses Collagen Synthesis and Type III Procollagen mRNA Levels in Arterial Smooth Muscle Cell Cultures",** Arteriosclerosis and Thrombosis 1993: 13: pages 680-686.

59. Mayani H, **"Composition and Function of the Haematopoietic Microenvironment in Human Myeloid Leukaemia",** Leukaemia 1996: 10: pages 1041-1047.

60. Mayani H, Guilbert LJ, Clark SC, et al, **"Composition and Functional Integrity of the In Vitro Haematopoietic Microenvironment in Acute Myelogenous Leukaemia: Effects of Macrophage Colony-Stimulating Factor",** Experimental Haematology 1992: 20: pages 1077-1084.

61. Mier W, Razelle K, Zeev E, et al, **"Cytokine Expression in Adherent Layers from Patients with Myelodysplastic Syndrome and Acute Myelogenous Leukaemia",** Leukaemia Research 1995: 19: pages 23-24.

62. Menzel T, Rahman Z, Calleja E, et al, **"Elevated Intracellular Levels of Basic Fibroblast Growth Factor Correlates with Stage of Chronic Lymphocytic Leukaemia and is Associated with Resistance to Fludarabine",** Blood 1996: 87: pages 1056-1063.

63. Metcalf D, Pre-progenitor cells: **"A Proposed New Category of Haematopoietic Precursor Cells",** Leukaemia 1998: 12: pages 1-3.

64. Michael RB, **"The Risk of Secondary Leukaemia Following Autologous Stem Cell Transplantation",** Leukaemia Research 1999: 23: pages 281-282.

65. Mitsuhiro M, Yasuhiro M, Yoshiyasu S, et al, **"Establishment of a Myelodysplastic Syndrome (MDS)/ Secondary AML-Derived T-Lymphoid Cell Line K2-MDS",** Leukaemia Research 1999: 24: pages 103-108.

66. Monroy RL, Davis TA, Nielsen TB, et al, **"Peripheral Blood Haematopoietic Progenitor/Stem Cells Proliferate to Form Colonies in Liquid Culture but Require Contact with Vascular Endothelial Cells and GM-CSF",** International Journal of Cell Cloning 1992: 10: pages 105-115.

67. Moore KA, Ema H, Lemischka IR, **"In Vitro Maintenance of Highly Purified, Transplantable Haematopoietic Stem Cells",** Blood 1997: 89: pages 4337-4347.

68. Muench MO, Roncarolo MG, Rosnet O, et al, **"Colony Forming Cells Expressing High Levels of CD34 are the Main Targets for Granulocyte Colony Stimulating Factor in the Human Fetal Liver",** Experimental Haematology 1997: 25: pages 277-287.

69. Novitzky N, Kruger W, Mohammed R, **"Cryopreservation of the Bone Marrow From Patients with Acute Myeloid Leukaemia Leads to Functional Abnormalities",** European Journal of Haematology 1999: 62: pages 83-89.

70. Novitzky N, Mohammed R, **"Alterations in Both the Haematopoietic Microenvironment and the Progenitor Cell Population Follow the Recovery from Myeloablative Therapy Bone Marrow Transplantation",** Experimental Haematology 1995: 23: pages 1661-1666.

71. Novitzky N, Peter J, **"Immunosuppressive Therapy in Bone Marrow Aplasia: The Stroma Functions Normally to Support Haematopoiesis",** Experimental Haematology 1995: 23: pages 1472-1477.

72. Novitzky N, Mohammed R, **"Alterations in the Progenitor Cell Population Follow Recovery from Myeloablative Therapy and Bone Marrow Transplantation"**, Experimental Haematology 1997: 25: pages 471-477.

73. O'Flatery E, Sparrow R, Szer J, **"Bone Marrow Stromal Function from Patients after Bone Marrow Transplantation",** Bone Marrow Transplantation 1995: 15: pages 207-212.

74. Ogawa M, **"Differentiation and Proliferation of Haematopoietic Stem Cells",** Blood 1993: 11: pages 2844-2853.

75. Okunief P, Barrett AJ, Phang SE, et al, **"Circulating Basic Growth Factor Declines During Cy/TBI Bone Marrow Transplantation",** Bone Marrow Transplantation 1999: 23: pages 1117- 1120.

76. Olivier LJ, Rifkin DB, Gabrilove J, et al, **"Long-Term Culture of Human Bone Marrow Stromal Cells in the Presence of Basic Fibroblast Growth Factor",** Growth Factor 1990: 3: pages 231-236.

77. Perkins S, Fleishman RA, **"Stromal Cell Progeny of Murine Bone Marrow Fibroblast Colony Forming Units are Clonal Endothelial-Like Cells that Express Collagen IV and Laminin",** Blood 1990: 75: pages 620-625.

78. Petros WP, Rabinowitz J, Stuart AR, et al, **"Disposition of Recombinant Human Granulocyte-Macrophage Colony Stimulating Factor in Patients Receiving High Dose Chemotherapy and Autologous Bone Marrow Support",** Blood 1992: 80: pages 1135-1140.

79. Quito FL, Beh J, Bashayan O, et al, **"Effects of Fibroblast Growth Factor-4 on Long-Term Cultures of Human Bone Marrow Cells",** Blood 1996: 87: pages 1282-1291.

80. Roingeard F, Binet C, Lecron J, et al, **"Cytokines Released In Vitro by Stromal Cells from Autologous Bone Marrow Transplant Patients with Lymphoid Malignancy",** European Journal of Haematology 1998: 61: pages 100-108.

81. Rosendaal M, Hodgson GS, Bradley TR, **"Organization of Haematopoietic Stem Cells: The Generation-Age Hypothesis",** Cell Tissue Kinetics 1979: 12: pages 17-29.

82. Sandhaus LM, Edinger MG, Tubbs RR, et al, **"A Simplified Method of $CD34^+$ Cell Determination for Peripheral Blood Progenitor Cell Transplantation and Correlation with Clinical Engraftment",** Experimental Haematology 1998: 26: pages 73-78.

83. Santo GW, **"Bone Marrow Transplantation in Haematological Malignancies",** Cancer 1990: 65: page 786.

84. Sato N, Sawada K, Koizumi K, et al, **"In Vitro Expansion of Human Peripheral Blood $CD34^+$ Cells",** Blood 1993: 82: pages 3600-3609.

85. Sensebe L, Li J, Lilly M, et al, **"Non-Transformed Colony Derived Stromal Cell Lines from Normal Human Marrows. I. Growth Requirement and Myelopoiesis Supportive Ability",** Experimental Haematology 1995: 23: pages 507-513.

86. Shi Q, Rafii S, Wu MH, et al, **"Evidence for Circulating Bone Marrow Derived Cells",** Blood 1998: 92: pages 362-367.

87. Siczkowski M, Clarke D, Gordon MY, **"Binding of Primitive Haematopoietic Progenitor Cells to Marrow Stromal Cells Involves Heparan Sulphate",** Blood 1992: 80: pages 912-919.

88. Simon PH, James AW, Kodetthoor BU, ET AL, **"Morphological Characterization of Stromal Cell Types in Haematologically Active Long-Term Murine Bone Marrow Cultures",** The Journal of Histochemistry and Cytochemistry 1995: 43: pages 371- 379.

89. Simmons P, Zannettino A, Grontos S, et al, **"Potential Adhesion Mechanisms for Localization of Haematopoietic Progenitors to Bone Marrow Stroma",** Leukaemia and Lymphoma 1994: 12: pages 353-363.

90. Simmons PJ, Torok-Storb B, **"Identification of Stromal Cell Precursors in Human Bone Marrow by a Novel Monoclonal Antibody, STRO-1",** Blood 1991: 78: pages 55-62.

91. Sitnicka E, Wang Q, Schickwann, T, et al, **"Support versus Inhibition of Haematopoiesis by Two Characterized Stromal Cell Types",** Stem Cells 1995: 13: pages 655-665.

92. Sparrow RL, O"Flattery E, Blanksby TM, et al, **"Perturbation in the Ability of Bone Marrow Stroma from Patients with Acute Myeloid Leukaemia but not Chronic Myeloid Leukaemia to Support Normal Early Haematopoietic Progenitor Cells",** Leukaemia Research 1997: 21: pages 29-36.

93. Stolze B, Emmendorffer A, Corbacioglu S, et al, **"Effects of Bone Marrow Fibroblasts on the Proliferation and Differentiation of Myeloid Leukaemic Cell Lines",** Experimental Haematology 1995: 23: 1387-1387.

94. Strobel E, Gay RE, Greenberg PL, **"Characterization of the In Vitro Stromal Microenvironment of Human Bone Marrow",** International Journal of Cell Cloning 1986: 4: pages 341-356.

95. Strobel E, Mobest D, Von Kleist S, et al, **"Adhesion and Migration are Differentially Regulated in Haematopoietic Progenitor Cells by Cytokines and Extracellular Matrix",** Blood 1997: 90: pages 3524-3532.

96. Sundararajan VM, Alan WF, Howard WT, "**Maintenance of CD34 Expression During Proliferation of $CD34^+$ Cord Blood Cells on Glycosylaminoglycan Surface",** Stem Cells 1999: 17: pages 295-305.

97. Suzuki T, Muroi K, Tomizuka H, et al, **"Characterization of Enriched $CD34^+$ Cells from Healthy Volunteers and those From Patients Treated with Chemotherapy plus Granulocyte Colony Stimulating Factor",** Stem Cells 1995: 13: pages 273-280.

98. Takahashi GW, Moran D, Andrew DF, et al, "**Differential Expression of Collagenase by Human Fibroblast and Bone Marrow Stromal cells",** Leukaemia 1994: 8: pages 305-308.

99. Thorpe R, Wadhwa M, Bird CR, et al, **"Detection and Measurement of Cytokines",** Blood Reviews 1992: 6: pages 133-148.

100. Timeus F, Crescenzio N, Basso G, et al, **"Cell Adhesion Molecule Expression in Cord Blood $CD34^+$ cells",** Stem Cells 1998: 16: pages 120-126.

101. Wang X, Fjerdingstad H, Strom-Gundersen I, et al, **"Maturation Rate of Mouse Neutrophilic Granulocytes: Acceleration by Retardation of Proliferation, but no Detectable Influence from G-CSF or Stromal cells",** Stem Cells 1999: 17: pages 253-264.

102. Warren SA, Nicos AN, **"Haematopoietic Growth Factor Receptor Abnormalities in Leukaemia",** Leukaemia Research 1998: 22: pages 1097-1111.

103. Wilson EL, Rifkin BD, Kelly F, et al, **"Long-Term Culture of Human Bone Marrow Stromal Cells in the Presence of Basic Fibroblastic Growth Factor",** Growth Factors 1990: Vol. 3: pages 231-236.

104. Yuen D, Mittal L, Deng C, et al, **"Generation of a Primitive Cell Line and Promotion of its Growth by Basic Fibroblast Growth Factor",** Blood 1998: 91: pages 3202-3209.

105. Zanjani ED, Almeida-Porada G, Livingstone AG, et al, **"Human Bone Marrow $CD34^-$ Cells Engraft InVivo and undergo Multilineage Expression that Includes Giving Rise to $CD34^+$ Cells",** Experimental Haematology 1998: 26: pages 353-360.

APPENDIX A

REAGENTS

3% Agar

- 6g of commercially bought agar was dissolved in 200 ml of distilled water and brought to boil until it was clear and with bubbles. It was autoclaved at 115°C for 10 minutes. Volumes of 3 ml were aliquot in to bottles and stored at room temperature until use.

AB Serum

- This was supplied by the Western Province Blood Transfusion Services and aliquot in to 5 ml Cryopreservation tubes and stored at -70°C until use.

0.83% Ammonium Chloride (NH_4CL)

- 2.49g of ammonium chloride was dissolved in 300 ml of distilled water and boiled for 10 minutes. It was autoclaved at 115°C.

Alpha Minimal Essential Medium (α-MEM)

- One vial was mixed with 2.2g of sodium bicarbonate (NaHCO3) and dissolved in 1000 ml of distilled water. The mixture was stirred until the pH was between 7.1-7.25 and filtered using 0.2 µmm diameter media caps into 200 ml volumes and stored in 4°C fridge.

Basic Fibroblast Growth Factor (bFGF)

1 mg/ml stock was diluted with sterile phosphate buffered saline (PBS) in to 100 and 1000 ng concentrations and stored at 4°c.

Buffer (pH 6.8)

- One tablet was dissolved in 1000 ml of distilled water and stored at room temperature.

Campath 1M

- It was commercially bought in a vial of 2 ml volume and stored at -70°C.

Foetal Calf Serum (FCS)

- This was also bought from Gibco and aliquot in to 15 ml volumes and stored in -70°C.

Gentamycin (1:5 dilution)

- 2 ml of gentamycin was diluted with 8 ml of commercially bought sterile water, aliquot into 0.5 ml volumes and stored at 4°C.

Giemsa for staining CFU-F dishes

- 2 ml of Giemsa stain was diluted with 70 ml of pH buffer and filtered using a 150 mm filter paper.

Giemsa for staining slides

- 2 ml of Giemsa stain was diluted with 140 ml of pH 5.8 buffer and filtered using a filter paper.

Glyteraldehyde (0.25%)

- 5 ml of glyteraldehyde was diluted with pH 6.8 buffer to the 50 ml mark in a fat tube.

Heparan Sulphate

- 0.5 mg/ml powder was dissolved in distilled water to get concentration of 200 ng/ml and stirred on a magnetic stirrer. It was autoclaved at 115°C and cooled down before it was stored in 4°C.

Horse Serum

- It was commercially bought from Gibco, aliquot into 15 ml volumes and stored at -70°C until use.

Hydrocortisone

- One vial was reconstituted with 2 ml of commercially bought sterile water, aliquot in to 0.1 ml volumes and stored at -70°C.

Iscove' Modified Dulbecco Medium (IMDM)

- One vial mixed with 3.2g of sodium bicarbonate (NaHCO3) was dissolved in 1000 ml of distilled water. It was filter using media cap of 0.2 µmm pore diameter in to storing bottles at 4°C. One sample was sent to the Microbiology laboratory to check for any bacteria growth and another sample was left in 37°C incubator for 3 days.

May-Grünwald for staining CFU-F

- Prepared by weighing 5g of May-Grünwald powder and transferred in to a conical flask of 5000 ml volume and filled up to the mark with methanol. The mixture was placed on a stirrer for 1 hour and then put in a water bath of 56°C for 15 minutes. The mixture was allowed to stand for 24 hours with occasional mixing and filtered using a filter paper. This is a modified method by Dacie J.V, and Lewis S.M, Practical Haematology, 8th Edition, 1995, page 85.

May Grünwald for staining slides

- 20 ml of the stain was diluted with 150 ml of buffer pH 5.8 and filtered using a filter paper.

Phosphate Buffered Saline (PBS)

- Three tablets of buffered saline were dissolved in 300 ml of distilled water and put on the mixer. It was sent for autoclaving.

5% Trypsin

- One vial of trypsin powder was reconstituted with 10 ml of commercially bought sterile water when it was ready to be used.

APPENDIX B

Age group of normal individuals

CONTROLS	AGE	GENDER
Control 1	57	Male
Control 2	27	Male
Control 3	39	Male
Control 4	68	Male
Control 5	29	Male
Control 6	58	Male
Control 7	41	Female
Control 8	68	Male
Control 9	67	Male
Control 10	48	Female
Control 11	74	Female
Control 13	44	Female
Control 15	43	Male
Control 16	35	Female
Control 17	46	Male
Control 18	19	Female
Control 19	32	Male
Control 20	61	Male
Control 21	45	Female
Control 22	64	Male
Control 23	67	Female
Control 24	48	Female
Control 25	43	Female
Control 26	70	Female
Control 27	68	Female
Control 28	43	Female
Control 29	71	Male
Control 30	52	Female
Control 31	50	Male
Control 32	42	Male
Control 33	57	Female
Median	48	
Ranges	19-74	

Fibroblastic colony results of normal individuals

CFU-Fs RESULTS

A= BLANK
B=0.2 FGF+HS
C= 0.2 FGF
D= 2 FGF+ HS
E= 2 FGF
F= 20 FGF+ HS
G= 20 FGF

The primary and secondary colony forming unit- fibroblasts (CFU-Fs) were established at the concentrations shown below in 80 mm petri dishes. The cells were cultured with 20% FCS/---MEM FOR 3 or 9 days at concentrations shown below. Plates were stained with May -Grunwald- Giemsa.

Plating concentration: primary = 1 x 106/5ml (9 DAYS)
Secondary = 0.05 x 106/5ml (3 DAYS)

NAMES	A	B	C	D	E	F	G
Control # 8 primary	30	39	17	36		32	16
Control # 9 primary	53	67			34	37	19
Control # 10 primary	18	21	20	15		10	
Control # 11 primary	23	22	11	23	11	7	5
Control # 13 primary	14	24	19	14	13	11	7
Control # 14 primary		3	23		14	9	12
Control # 15 primary	31						
Control # 17 primary	19	23	12	20	17	26	16
Control # 20 primary	30	22	21	22	18	26	16
Control # 21 primary	32	24		23	23	32	27
Control # 22 primary	33	35	25	36	29	40	28
T-test		0.9625756	0.1403264	0.1582157	0.3791422	0.5534169	0.1832134
Paired t-test		0.3064629	0.2737201	0.140794	0.0547473	0.1587879	0.0066924
Ranges	14-53	3.0-67	12.0-25	14-36	11.0-34	7.0-40	5.0-28

Blastic colony results of normal individuals

BLASTIC COLONIES RESULTS

A= BLANK
B=0.2 FGF+HS
C= 0.2 FGF
D= 2 FGF+ HS
E= 2 FGF
F= 20 FGF+ HS
G= 20 FGF

1 x 104 selected cells were layered onto stroma that has been supplemented with FGF and HS for 21 days. The cells were incubated for 2 hours for optimal adherence.
Non- adhered cells were removed and cultured in CFU-GM assay.

Plated at 1 x 104/ml

NAMES	A	B	C	D	E	F	G
Control # 2 primary	154	159	218	260	128	271	146
Control # 3 primary	3	21	62	51	60	43	45
Control # 6 primary							
Control # 11 primary	10	48	16	41	7	24	8
Control # 21 primary	138	140	113	144	131	177	137
Control # 24 primary	139	112	103	91	81	169	99
Control # 25 primary	184	102	99	93		113	71
Control # 26 primary	172	135	121	197	168	238	207
Control # 27 primary	174	115	117	185	176	219	203
T-test		0.5724658	0.9365776	0.446271	0.4954703	0.241792	0.316814
Paired t-test		0.2568907	0.8563509	0.0659778	0.1278488	0.0286962	0.0198601
Ranges	3.0-184	21-159	16-218	41-260	7-176	43-271	8-207

Control CD34+ cells on to control stroma (without bFGF treatment)

Controls	CFU-bl x 10^4/ml	CFU-GMs
Control #17	90	80
Control #20	103	258
Control #24	130	337
Control #22	84	141
Control #26	139	126
Control #31	72	167
Control #33	24	249

Stromal Cell Numbers

A= BLANK
B=0.2 FGF+HS
C= 0.2 FGF
D= 2 FGF+ HS
E= 2 FGF
F= 20 FGF+ HS
G= 20 FGF

0.5×10^6 cells cultured with different FGF concentrations, in medium supplemented with 15% FCS, 2×10^{-7} M
A suspension obtained by incubation of stromal cells with 5% trypsin for 10 minutes and inactivated by addition of cold 4C FCS, and cells counted on the particle counter.

Counts expressed as $\times 10^6$/ml

CONTROLS	A	B	C	D	E	F	G
Control # 1	0.6	0.54	0.45	0.75	0.4	1.04	0.52
Control # 2	0.57	0.29	0.9	1.19	1.16	1.89	1.61
Control # 3	0.15	0.25	0.35	0.38	0.39	0.8	0.33
Control # 4	0.34	0.15	0.4	0.97	0.29	0.44	0.98
Control # 5	1.3	0.12	0.94	0.28	1.2	0.46	1.52
Control # 6	0.45	0.81	1.99	4.11	1.14	0.61	1.11
Control # 8	0.33	0.35	0.27	0.65	0.45	0.88	0.43
Control # 11	0.43	0.96	1.1	1.64	0.91	3.92	1.42
Control # 13	0.76	0.52	0.56	1.1	0.85	1.3	0.93
Control # 14	0.27	0.24	0.32	0.27	0.35	0.34	0.51
Control # 15	1.66	1.45	1.38	1.41	1.36	2.6	1.47
Control # 22	0.34	1.67	1.24	2.71	1.69	3.3	1.76
Control # 23	0.76	0.56	0.95	1.26	0.55	1.53	1.55
T-test		0.9832539	0.2610448	0.1836795	0.1680489	0.0731182	0.2841629
Paired t-test		0.9808519	0.0828389	0.036821	0.0897597	0.0327583	0.168224
Median							
Ranges	0.15-1.66	0.12-1.67	0.32-1.99	0.27-4.11	0.29-1.36	0.44-1.89	0.33-1.76

Confluence of normal individual at 3 weeks

	Confluence @ 3 weeks (%)						
	Blank	0.2ng + HS	0.2ng	2ng + HS	2ng	20ng + HS	20ng
Control 1	60	45	45	65	60	85	85
Control 2	55	50	50	75	65	90	90
Control 3	40	40	30	55	50	70	70
Control 4	55	45	50	65	60	75	75
Control 5	65	35	30	70	60	95	98
Control 6	30	50	50	80	65	86	75
Control 8	50	50	55	80	80	90	70
Control 9	55	60	60	85	80	90	90
Control 11	65	45	45	95	70	80	70
Control 13	60	50	55	70	70	95	80
Control 15	60			70		79	
Control 17	50			65		80	
Control 20	45			85		99	
Control 22	60			50		95	
Control 24	50			60		60	
Control 26	45			70		75	
Control 29	50			65		65	
Control 30	50			75		70	
Control 30	65			80		90	
Control 31	40			70		75	
Control 32	55			70		65	
Control 33	55			65		70	
Median	55	47.5	50	70	65	80	77.5
T test		0.08250223	0.11695085	1.42432E-07	0.00060715	0.00489634	1.0847E-08
Paired t test						6.7574E-10	
Ranges	30-65	35-60	30-60	50-95	50-80	65-99	70-98

Stromal cell numbers and CFU-F results of patients

BMT PATIENTS RESULTS								
Patients	**Diagnosis**	**BMT**	**Stromal cell numbers x 10^6/ 2ml**			**CFU-Fs per 10^6 /5ml**		
			Blank	2ng	20ng	Blank	2ng	20ng
Patient 1	AML-M2	Allo	0.28	0.25	0.46	6	6	5
Patient 2	NHL	Allo	0.96	2.03	2.42	12	14	13
Patient 3	CML	Allo	0.43	0.72	1.14	n/a		
Patient 4	NHL-Cat D	Allo	1.2	1.6	2.5	19	18	28
Patient 5	NHL-Cat C	Allo	0.53	1.29	1.79	23	24	24
Patient 6	ALL	Allo	1.54	2.8	3.35	28	33	33
Patient 7	AML	Auto	0.64	1.75	2.65	7	8	8
Patient 8	NHL	Allo	0.28	0.67	2.02	7	6	8
Patient 9	NHL	Allo	0.6	0.52	1.03	19	18	17
Patient 10	SAA	Allo	1.73	2.01	2.2	23	24	25
Patient 11	NHL	Allo	N/a			6	7	4
Patient 13	ALL	Allo	0.67	0.93	1.41	7	12	10
Patient 14	Bl-NHL	Allo	1.5	2.64	3.43	5	4	5
Patient 15	MDS	Allo	1.33	1.89	2.54	6	5	8
Patient 16	NHL	Allo	0.65	0.9	1.38	7	3	5
Median values			0.66	1.445	2.11	7	10	9
T test				0.03773	0.07075		0.8797	0.82871
Paired t test				0.0005	0.00023 8.2E-06		0.45146	0.1326
Ranges			0.28-1.73	0.25-2.8	0.46-3.43	5.0-28.0	3.0-33.0	4.0-33.0

Demographic Data of Patients

PATIENTS	AGE	GENDER	DIAGNOSIS	Time post-Tx
Patient 1	46	Male	AML-M2	12 months
Patient 2	37	Male	NHL	6 months
Patient 3	29	Male	CML	6 months
Patient 4	52	Female	AML	12 months
Patient 5	48	Male	NHL-Cat-C	11 months
Patient 6	17	Male	ALL	12 months
Patient 7	43	Male	AML-M2	6 months
Patient 8	37	Male	NHL	6 months
Patient 9	57	Female	NHL	6 months
Patient 10	17	Male	SAA	6 months
Patient 11	51	Female	NHL	6 months
Patient 12	35	Male	AML	48 months
Patient 13	45	Female	ALL	6 months
Patient 14	23	Male	NHL	6 months
Patient 15	42	Male	MDS	6 months
Patient 16	41	Male	NHL	6 months
Median	41.5			
Ranges	17-57			

Clinical Data of Transplantation Patients

Patients	Infused $CD34^+ \times 10^6$/kg	Selected $CD34^+$ (%)	Infused MNC $\times 10^8$/kg	CFU-GM $\times 10^4$/kg
Patient 1	0.8	98.02	5.3	148.6
Patient 2	0.29	INS	4.69	3.1
Patient 3	0.83	INS	6.97	0
Patient 4	0.55	82.93	4.2	3.36
Patient 5	0.92	INS	6.39	6.4
Patient 6	0	66.38		106.5
Patient 7	0.77	INS	6.16	35.8
Patient 8	3.76	92.6	3.76	12.9
Patient 9	2.67	97.69	4.15	2.28
Patient 10	3.47	98.34	7.62	24.4
Patient 11	2.61	83.96	8.68	38.8
Patient 12	0	INS		0
Patient 13	6.47	98.36	11.59	56.7
Patient 14	6.2	81.9	8.71	379.7
Patient 15	13.57	84.47	13.02	83.3
Patient 16	3.08	98.48	9.81	33.3
Median	1.765	92.6	6.68	28.85
T Test			0.000987362	
Ranges	0.29-13.57	66.38-98.48	3.76-13.02	3.1-148.6

Control CD34+ cells onto patient stroma (corrected for surface area)

Patient	Diagnosis	BMT	Day 6 col x 10^4/ml			Day 9 col x 10^4/ml			Day 11 col x 10^4/ml			CFU-GM		
			Blank	2ng	20ng	Blank	2ng	20ng	Blank	2ng	20ng	Blank	2ng	20ng
Patient 1	AML-M2	Allo	11	15	19	15	52	41	20	39	58	181	187	239
Patient 2	NHL	Allo	84	58	91	90	64	95	74	89	71	242	406	445
Patient 3	CML	Allo	36	89	105	31	61	89	29	45	80	172	238	244
Patient 4	NHL-Cat D	Allo	23	31	46	11	21	23	8	16	21	19	22	27
Patient 5	NHL-Cat C	Allo	39	72	104	37	101	111	21	53	93	n/a	113	142
Patient 6	ALL	Allo	56	88	107	58	130	125	44	77	86	151	166	197
Patient 7	AML	Allo	68	60	97	77	30	107	46	35	52	221	267	299
Patient 8	NHL	Allo	27	45	26	23	46	26	24	41	19	56	85	44
Patient 9	NHL	Allo	16	11	6	16	21	n/a	17	n/a	n/a	71	65	62
Patient 10	SAA	Allo	39	94	133	48	99	141	46	83	139	209	176	137
Patient 13	ALL	Allo	6	0	7	6	7	9	3	2	7	92	114	170
Patient 15	MDS	Allo	72	111	113	82	109	110	n/a	n/a	n/a	25	16	45
Patient 16	NHL	Allo	55	68	53	68	66	71	68	70	84	55	46	49
Median			39	60	91	37	61	92	26.5	45	71	121.5	114	142
T test				0.1848	0.05347		0.17054	0.02449		0.1264	0.02615		0.58358	0.3871
						0.82941	0.7304	0.60967		0.43013	0.59233			
Paired T test				0.0375	0.00799		0.06825	0.00376		0.00744	0.0122			
Ranges			6.0-84	11.0-111	6.0-133	6.0-90	7-130	9-141	3.0-74	2.0-89	7.0-139	19-242	16-406	27-299

Appearance of Stroma at 3 weeks

Patients	Diagnosis	BMT	Culture	Blank (%)	2ng (%)	20ng (%)
Patient 1	AML-M2	Auto	Blank	45	70	98
Patient 2	NHL	Allo	Blank	50	65	90
Patient 3	NHL	Allo	Blank	20	70	85
Patient 4	NHL- Cat D	Allo	Blank	40	70	95
Patient 5	NHL-Cat C	Allo	Blank	20	70	92
Patient 6	ALL	Allo	Blank	50	65	80
Patient 7	AML	Auto	Blank	55	80	90
Patient 8	NHL	Auto	Blank	45	60	76
Patient 9	NHL	Auto	Blank	30	50	75
Patient 10	SAA	Allo	Blank	50	75	89
Patient 11	NHL	Allo	Blank	10	25	30
Patient 12	ALL	Allo	Blank	0	0	20
Patient 13	ALL	Allo	Blank	30	40	55
Patient 14	BL-NHL	Allo	Blank	40	55	70
Patient 15	MDS		Blank	45	60	65
Patient 16	NHL		Blank	30	45	40
Median				40	62.5	78
T test					0.0028049	0.0578237
Paired T test					1.142E-05	1.645E-05 4.203E-07
Ranges				0-55	0-80	20-98

Patient $CD34^+$ cells onto control stroma (corrected for surface area)

PATIENT	Diagnosis	BMT	Day 6 col x 10^4/ml			Day 9 col x 10^4/ml			Day 11 col x 10^4/ml			CFU-GM		
			Blank	2ng	20ng	Blank	2ng	20ng	Blank	2ng	20ng	Blank	2ng	20ng
Patient 1	AML-M2	Allo	71	90	139							21	84	97
Patient 2	NHL	Allo												
Patient 3	CML	Allo												
Patient 4	NHL-Cat D	Allo	121	193	228	130	201	255	92	205	262	207	331	423
Patient 5	NHL-Cat C	Allo	15	71	85	23	67	92	17	48	51	51	56	27
Patient 6	ALL	Allo	47	83	109	61	93	124	50	87	102	11	98	243
Patient 7	AML	Allo	89	124	159	98	n/a	n/a				96	193	106
Patient 8	NHL	Allo	71	101	165	79	154	196	69	148	166	70	121	158
Patient 9	NHL	Allo	56	119	179	59	151	196	63	167	201			
Patient 10	SAA	Allo	31	58	69	40	74	97	36	90	102	118	147	204
Patient 11	NHL	Allo	44	58	83	31	78	96				215	199	250
Patient 12	ALL	Allo	8	21	n/a	15	26	n/a	10	16	n/a	108	51	31
Patient 13	ALL	Allo	8	21	6	15	14	16	44	46	46	83	33	13
Patient 14	BL-NHL	Allo	36	51	70	37	61	86	36	51	58	45	43	35
Patient 15	MDS	Allo	14	14	15	21	27	27	21	25	27	5	24	14
Patient 16	NHL	Allo	3	6	5	5	6	6	3	6	4	118	41	34
Median			40	64.5	85	37	70.5	96	36	51	80	83	84	97
T test				0.1011	0.2343		0.1232	0.3386		0.073	0.526		0.5024	0.7018
Paired T test				0.0004	0.0026		0.0017	0.0015		0.0085	0.033		0.2465	0.3398
					0.0004			0.0016			0.0119			0.2071
							0.0021	0.0796		0.0107	0.0254		0.643	0.4937
Ranges			3.0-121	6.0-193	5.0-228	5.0-130	6.0-201	6.0-255	3.0-92	6.0-205	4.0-262	5.0-215	24-331	13-423